(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 409 815 A1

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 90870111.3

(22) Date of filing: 16.07.90

(51) Int. Cl.5: C12N 15/54, C12N 9/10, C12N 15/82, C12N 5/04, A01H 1/00, C07H 21/04

(30) Priority: 17.07.89 US 380963

(43) Date of publication of application: 23.01.91 Bulletin 91/04

(84) Designated Contracting States: GR

(71) Applicant: MONSANTO COMPANY
Patent Department 800 North Lindbergh Boulevard
St. Louis, Missouri 63167(US)

(72) Inventor: Eichholtz, David Alan
1718 Preston Place
St. Louis, Missouri 63104(US)
Inventor: Kishore, Ganesh Murthy
15354 Grantley Drive
Chesterfield, Missouri 63017(US)
Inventor: Gasser, Charles Scott
3301 Seabright Avenue
Davis, California 95616(US)

(74) Representative: Lunt, John Cooper et al
Monsanto Services International S.A. Patent Department Avenue de Tervuren 270/272
L.Box 21
B-1150 Brussels(BE)

(54) Glyphosate-tolerant 3-enolpyruvyl-3-phosphoshikimate synthases.

(57) Glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthases, DNA encoding glyphosate-tolerant EPSP synthases, plant genes encoding the glyphosate-tolerant enzymes, plant transformation vectors containing the genes, transformed plant cells and differentiated transformed plants containing the plant genes are disclosed. The glyphosate-tolerant EPSP synthases are prepared by substituting an alanine residue for a glycine residue in a first conserved sequence found between positions 80 and 120, and either an aspartic acid residue or asparagine residue for a glycine residue in a second conserved sequence found between positions 120 and 160 in the mature wild type EPSP synthase.

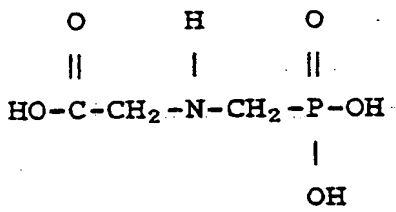
EP 0 409 815 A1

GLYPHOSATE-TOLERANT 5-ENOLPYRUVYL-3-PHOSPHOSHIKIMATE SYNTHASES

BACKGROUND OF THE INVENTION

Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. It is now possible to produce plants which have unique characteristics of agronomic importance. Certainly, one such advantageous trait is herbicide tolerance. Herbicide-tolerant crop plants could reduce the need for tillage to control weeds, thereby effectively reducing costs to the farmer.

One herbicide which is the subject of much investigation in this regard is N-phosphonomethylglycine.



This herbicide is a non-selective, broad spectrum, postemergence herbicide which is registered for use on more than fifty crops. This molecule is an acid, which dissociates in aqueous solution to form phytotoxic anions. Several anionic forms are known. As used herein, the term "glyphosate" refers to the acid and its anions.

Glyphosate inhibits the shikimic acid pathway which provides a precursor for the synthesis of aromatic amino acids. Specifically, glyphosate inhibits the conversion of phosphoenolpyruvate and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimate synthase.

It has been shown that glyphosate-tolerant plants can be produced by inserting into the genome of the plant the capacity to produce a higher level of EPSP synthase.

The present invention provides a means of enhancing the effectiveness of glyphosate-tolerant plants by producing variant EPSP synthase enzymes which exhibit a lower affinity for glyphosate while maintaining catalytic activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequences for EPSP synthase enzymes from various plant, and bacterial species.

Figure 2 represents a map of plasmid pMON8135.

Figure 3 represents a map of plasmid pMON895.

Figure 4 represents a map of plasmid pMON915.

Figure 5 represents a restriction map of the T-DNA of pTiT37 plasmid of *A. tumefaciens* A208.

Figure 6 represents a map of intermediate plant transformation vector pMON987.

Figure 7 represents a map of plasmid pMON8631.

STATEMENT OF THE INVENTION

The present invention provides novel EPSP synthase enzymes which exhibit increased tolerance to glyphosate herbicide while maintaining low K_m values for phosphoenolpyruvate. The subject enzymes of this invention have an alanine for glycine substitution and an aspartic acid for glycine substitution as described hereinafter.

In another aspect, the present invention provides a method for the isolation of amino acid substitutions which will maintain a high level of glyphosate tolerance while lowering the K_m values for phosphoenol-

pyruvate.

All peptide structures represented in the present specification and claims are shown in conventional format wherein the amino group at the N-terminus appears to the left and the carboxyl group at the C-terminus at the right. Likewise, amino acid nomenclature for the naturally occurring amino acids found in protein is as follows: alanine (ala;A), asparagine (Asn;N), aspartic acid (Asp;D), arginine (Arg;R), cysteine (Cys;C), glutamic acid (Glu;E), glutamine (Gln;Q), glycine (Gly;G), histidine (His;H), isoleucine (Ile;I), leucine (Leu;L), lysine (lys;K), methionine (Met;M), phenylalanine (Phe;F), proline (Pro;P), serine (Ser;S), threonine (Thr;T), tryptophan (Trp;W), tyrosine (Tyr;Y) and valine (Val;V). For purposes of the present invention the term "mature EPSP synthase" relates to polypeptide without the N-terminal chloroplast transit peptide. It is now known that the precusor form of the EPSP synthase in plants (with the transit peptide) is expressed and upon delivery to the chloroplast, the transit peptide is cleaved yielding the mature EPSP synthase. All numbering of amino acid positions are given with respect to the mature EPSP synthase (without chloroplast transit peptide leader).

Figure 1 shows the amino acid sequence for EPSP synthase from various plant, and bacterial species. Inspection of the sequences and alignment to maximize the similarity of sequence reveals regions of conserved amino acid residues (indicated by the consensus sequence) in the regions where the alanine for glycine substitution and aspartic acid or asparagine for glycine substitution are made. Indeed, all monofunctional EPSP synthase enzymes reported in the literature and in the present specification reveal a glycine at the two positions in the conserved regions. Those familiar with the literature will recognize that some organisms, such as yeast and molds (Aspergillus sp) have a multifunctional "arom complex" which includes an EPSP synthase component. The noted amino acids are also conserved and introduction of the described substitutions in the EPSP synthase component of the multifunctional "arom complex" should also result in the glyphosate-tolerant activity.

Specifically, the glycine residue which is replaced by the alanine residue in the preparation of the glyphosate-tolerant EPSP synthases of the present invention occurs at position 96 in the EPSP synthase of *Bordetella pertussis* (Maskell et al., 1988); position 101 in the EPSP synthase of petunia; position 101 in the EPSP synthase of tomato; position 101 in the EPSP synthase of *Arabidopsis thaliana*; position 101 in the EPSP synthase of *Brassica napus*; position 104 in the EPSP synthase of *Glycine max*; position 96 in the EPSP synthase of *E. coli* K-12 (Duncan et al., 1984) and position 96 in the EPSP synthase of *Salmonella typhimurium* (Stalker et al., 1985). The glycine residue which is replaced by an amino acid residue selected from the group consisting of aspartic acid and asparagine in the preparation of the glyphosate-tolerant EPSP synthases of the present invention occurs at position 137 in the EPSP synthase of *Bordetella pertussis*; position 144 in the EPSP synthase of petunia; position 144 in the EPSP synthase of tomato; position 144 in the EPSP synthase of *Arabidopsis thaliana*; position 144 in the EPSP synthase of *Brassica napus*; at position 147 in the EPSP synthase of *Glycine max*; position 137 in the EPSP synthase of *E. coli* K-12 and position 137 in the EPSP synthase of *Salmonella typhimurium*. These examples demonstrate that the alanine for glycine and aspartic acid for glycine replacements can be introduced into the conserved regions of these other wild-type EPSP synthase enzymes to yield glyphosate-tolerant EPSP synthase enzymes.

Hence, in one aspect the present invention provides glyphosate-tolerant EPSP synthase enzymes and a method for producing such enzymes which comprises substituting an alanine residue for the second glycine residue in a first conserved region having the sequence:

-L-G-N-A-G -T-A-

located between positions 80 and 120 in the mature wild-type EPSP synthase amino acid sequence, and substituting an amino acid residue selected from the group consisting of aspartic acid and asparagine for the terminal glycine residue in a second region having the sequence:

E-R-P-I-x_1-x_2-L-V-x_3-x_4-L-x_5-x_6-x_7-G -A

where x_1, x_2, x_3, x_4, x_6, and x_7 are any amino acid and x_5 is either arginine (R) or lysine (K) and said second region is located between positions 120 and 160 in the mature wild-type EPSP synthase amino acid sequence. In most cases the first conserved region will be located between positions 90 and 110 and the second conserved region between positions 135 and 150 in the mature EPSP synthase.

In one embodiment, glyphosate-tolerant EPSP synthase coding sequences are useful in further enhancing the efficacy of glyphosate-tolerant transgenic plants. Methods for transforming plants to exhibit glyphosate tolerance are disclosed in European Patent Office Publication No. 0218571 and commonly assigned U.S. patent application entitled "Glyphosate-Resistant Plants," Serial No. 879.814 filed July 7, 1986, the disclosures of which are specifically incorporated herein by reference. The present invention can be utilized in this fashion by isolating the plant or other EPSP synthase coding sequences and introducing the necessary change in the DNA sequence coding for EPSP synthase to result in the aforementioned

substitutions in the translated EPSP synthase enzyme.

In another aspect, the present invention provides a transformed plant cell and plant regenerated therefrom which contain a plant gene encoding a glyphosate-tolerant EPSP synthase enzyme having a first sequence:

-L-G-N-A-A-T-A-

located between positions 80 and 120 in the mature EPSP synthase amino acid sequence and having a second sequence:

$$E\text{-}R\text{-}P\text{-}I\text{-}x_1\text{-}x_2\text{-}L\text{-}V\text{-}x_3\text{-}x_4\text{-}L\text{-}x_5\text{-}x_6\text{-}x_7\text{-}\overset{D}{N}\text{-}A$$

where x_1,x_2,x_3,x_4,x_6, and x_7 are any amino acid, and x_5 is either arginine or lysine, said second sequence located between positions 120 and 160 in the mature EPSP synthase amino acid sequence. In most cases the first sequence will be located between positions 90 and 110 and the second sequence will be located between 135 and 150 in the mature EPSP synthase. The gene further comprises a DNA sequence encoding a chloroplast transit peptide attached to the N-terminus of the mature EPSP synthase coding sequence, said transit peptide being adapted to facilitate the import of the EPSP synthase enzyme into the chloroplast of a plant cell.

Therefore, in yet another aspect the present invention also provides a plant transformation or expression vector comprising a plant gene which encodes a glyphosate-tolerant EPSP synthase enzyme having a first sequence:

-L-G-N-A-A-T-A-

located between positions 80 and 120 in the mature EPSP synthase amino acid sequence and having a second sequence:

$$E\text{-}R\text{-}P\text{-}I\text{-}x_1\text{-}x_2\text{-}L\text{-}V\text{-}x_3\text{-}x_4\text{-}L\text{-}x_5\text{-}x_6\text{-}x_7\text{-}\overset{D}{N}\text{-}A$$

where x_1,x_2,x_3,x_4,x_6,and x_7 are any amino acid, and x_5 is either arginine or lysine, said second sequence located between positions 120 and 160 in the mature EPSP synthase amino acid sequence.

According to still another aspect of the present invention, a process is provided that entails cultivating such a plant and, in addition, propagating such plant using propagules such as explants, cuttings and seeds or crossing the plant with another to produce progeny that also display resistance to glyphosate herbicide.

The EPSP synthase sequences shown in Figure 1 represent a broad evolutionary range of source materials for EPSP synthases. These data demonstrate that EPSP synthase from bacterial and plant material contain the aforementioned conserved regions. However, those skilled in the art will recognize that a particular EPSP synthase may be produced by and isolated from another source material which may not have the exact sequence of the conserved region. Indeed, it has been found that an alanine may be inserted for the first glycine of the conserved region of petunia EPSP synthase with no attendant changes in glyphosate sensitivity.

While substituting either aspartic acid or asparagine for the glycine residue in the afore-described second conserved region results in a glyphosate resistant EPSP synthase, an aspartic acid substitution is most preferred. Those skilled in the art will recognize that substitutions of other amino acid residues are likely to yield EPSP synthase which are still glyphosate tolerant and possess a K_m sufficient to maintain catalytic activity. Hence, other substitutions at this position should be considered within the spirit and scope of the present invention.

The glyphosate-tolerant EPSP synthase plant gene encodes a polypeptide which contains a chloroplast transit peptide (CTP), which enables the EPSP synthase polypeptide to be transported into a chloroplast inside the plant cell. The EPSP synthase gene is transcribed into mRNA in the nucleus and the mRNA is translated into a precursor polypeptide (CTP/mature EPSP synthase) in the cytoplasm. The precursor polypetide is transported (imported) into the chloroplast at which time the CTP is cleaved to produce the mature EPSP synthase enzyme. Suitable CTPs for use in the present invention may be obtained from various sources. Most preferably, the CTP is obtained from the endogenous EPSP synthase gene of the subject plant to be transformed. Alternately, one may also use a CTP from an EPSP synthase gene of another plant. Although there is little homology between the CTP sequences of the EPSP synthase gene and the ssRUBISCO gene (see e.g., Broglie, 1983), one may find that non-homologous CTPs may function in particular embodiments. Suitable CTP sequences for use in the present invention can be easily

determined by assaying the chloroplast uptake of an EPSP synthase polypeptide comprising the CTP of interest as described hereinafter. It has been found that where a CTP is used other than the CTP of the EPSPS gene, one may need to include a small part of the N-Terminus of the source protein from which the CTP is derived to obtain efficient import of the EPSP synthase into the chloroplasts. In most cases, one would preferably isolate the EPSPS gene from the plant to be transformed and introduce the substitutions of the present invention into a cDNA construct made from the endogenous EPSPS mRNA of the subject plant. Suitable plants for the practice of the present invention include, but are not limited to, soybean, cotton, alfalfa, oil seed rape, flax, tomato, sugar beet, sunflower, potato, tobacco, maize, wheat, rice and lettuce.

Promoters which are known or found to cause transcription of the EPSP synthase gene in plant cells can be used in the present invention. Such promoters may be obtained from plants, plant pathogenic bacteria or plant viruses and include, but are not necessarily limited to, the 35S and 19S promoters of cauliflower mosaic virus and promoters isolated from plant genes such as EPSP synthase, ssRUBISCO genes and promoters obtained from T-DNA genes of *Agrobacterium tumefaciens* such as nopaline and mannopine synthases. The particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of glyphosate-tolerant EPSP synthase polypeptide to render the plant cells and plants regenerated therefrom substantially resistant to glyphosate. Those skilled in the art will recognize that the amount of glyphosate-tolerant EPSP synthase polypeptide needed to induce tolerance may vary with the type of plant.

The promoters used for expressing the EPSP synthase gene of this invention may be further modified if desired to alter their expression characteristics. For example, the CaMV35S promoter may be ligated to the portion of the ssRUBISCO gene which represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. As used herein, the phrase "CaMV35S" promoter includes variations of CaMV35S promoter, e.g. promoters derived by means of ligation with operator regions, random or controlled mutagenesis, addition or duplication of enhancer sequences, etc.

Variant EPSP synthases which contain only the glycine to alanine change at position 101 as described above are highly resistant to glyphosate. However, this resistance is accompanied by an increase in the binding constant (K_m) for phosphoenolpyruvate (PEP), one of the two natural substrates for the enzyme. The binding constant for the other substrate, shikimate-3-phosphate (S3P), is not affected. For example; the wild type petunia EPSP synthase has a binding constant (K_i) for the competitive inhibitor glyphosate of 0.4μM and a K_m for PEP of 5.2μM, while the variant form with the alanine for glycine substitution at position 101 has a K_i for glyphosate of 2000 μM and a K_m for PEP of 198 μM. Because of the elevated K_m for PEP normal catalytic activity will only be achieved at physiological concentrations of PEP by an elevated level of the variant enzyme. If a variant of EPSP synthase could be identified that had a high K_i for glyphosate and a lower K_m for PEP than the current variant, it would enhance the ability to achieve glyphosate tolerance in plant species or plant tissues where it is difficult to engineer a high level of gene expression. Selecting for new glyphosate-tolerant EPSP variants in bacteria would allow a much larger number of organisms to be screened in a shorter amount of time than would be possible in selections with higher organisms. The petunia EPSP synthase cDNA clone can be expressed in *E. coli* to produce a fully functional EPSP synthase enzyme when the cDNA clone is properly tailored for expression in *E. coli*. So, to hasten the isolation of variants a system for the expression and selection of variant forms of petunia EPSP synthase was developed in the common laboratory organism *E. coli*.

General Features of Selection Scheme for Identifying Glyphosate Resistant Variants with Low Km Values for PEP

Component	Features
E. coli Host	aroA- strain or prototrophic strain with endogenous EPSPS activity inhibited with low levels of glyphosate (empirically determined for each host).

Expression Plasmid	Self replicating plasmid with a weak bacterial promoter fused to a plant EPSPS gene, which is not able to complement the *E. coli* aroA mutation and support cell growth on minimal medium when expressed at a weak level. The promoter should be weak enough so that a fusion with a wild type EPSPS gene would be inhibited at a concentration of glyphosate similar to that needed to inhibit the endogenous bacterial EPSPS.
Mutagens	Should create point mutations, either single or multiple, transitions or transversions, but not insertions, deletions or frameshifts. Spontaneous mutations could also be selected, but would probably be less efficient.
Selection Medium	Minimal bacterial growth medium containing essential salts and minerals and sugars without aromatic amino acids. Antibiotics may be added which correspond to the drug resistance marker genes on the expression plasmid to select only for bacterial cells containing the expression plasmid. For aroA- hosts, glyphosate is not required to select for variants with low

K_m values for PEP, which are able to complement the *E. coli* aroA mutation when expressed at a weak level. For prototrophic *E. coli* strains, add glyphosate to inhibit the endogenous EPSPS activity.

Exemplary Heterologous Bacterial Expression/Selection System

A) Construction of pMON342 and pMON9566 expression vectors for wild type and variant petunia EPSP synthase (glycine (101) to alanine) in *E. coli.*

Plasmid pMON342 carries the "mature" wild-type petunia EPSP synthase coding sequence (without chloroplast transit peptide) expressed from tandem copies of the bacteriophage lambda pL promoter (double pL). This plasmid was derived from pMON9531 and pMON9556, petunia EPSP cDNA clones, as described below (the isolation of pMON9531 and pMON9556 is described hereinafter).

A unique NcoI site and ATG translational initiation signal were introduced at the amino terminus of the wild-type petunia EPSP synthase cDNA coding sequence for the mature protein. Simultaneously, the chloroplast transit peptide coding sequence was removed by subjecting M8017 (the M13mp9 clone of the 300 bp EcoRI cDNA fragment of pMON9531) to site directed mutagenesis using the procedure of Zoller and Smith (1983) and the following mutagenesis primer:

5′-ATCTCAGAAGGCTCCATGGTGCTGTAGCCA-3′

A variant phage clone was isolated that contained an NcoI site. The presence of the above-described mutation was confirmed by sequence analysis. This M13mp9 clone was designated M8019.

Plasmid pMON6001 is a derivative of pBR327 (Soberon et al., 1980) carrying the *E. coli* K12 EPSP synthase coding sequence expressed from two tandem copies of a synthetic bacteriophage lambda pL promoter. Plasmid pMON6001 was constructed in the following manner. First, pMON4 (Rogers et al., 1983) was digested with ClaI and the 2.5 kb fragment was inserted into a pBR327 plasmid vector that had also been cleaved with ClaI. The resulting plasmid, pMON8, contains the EPSP synthase coding sequence reading in the same direction as the beta-lactamase gene of pBR327.

To construct pMON25, a derivative of pMON8 with unique restriction endonuclease sites located adjacent to the *E. coli* EPSP synthase coding sequence, the following steps were taken. A deletion derivative of pMON4 was made by cleavage with BstEII and religation. The resultant plasmid pMON7 lacks the 2 kb BstEII fragment of pMON4. Next, a 150 bp HinfI to NdeI fragment which encodes the 5′ end of the EPSP synthase open reading frame was isolated after digestion of pMON7 with NdeI and HinfI and electroelution following electrophoretic separation on an acrylamide gel. This piece was added to the purified 4.5 kb BamHI-NdeI fragment of pMON8 which contains the 3′ portion of the EPSP synthase coding sequence and a synthetic linker with the sequence:

5′-GATCCAGATCTGTTGTAAGGAGTCTAGACCATGG-3′

3′-GTCTAGACAACATTCCTCAGATCTGGTACCTTA-5′

The resulting plasmid pMON25 contains the *E. coli* EPSP synthase coding sequence preceded by unique BamHI and BglII sites, a synthetic ribosome binding site, and unique XbaI and NcoI sites, the latter of which contains the ATG translational initiation signal of the coding sequence.

To construct pMON6001, pMON25 was digested with BamHI and mixed with a synthetic DNA fragment

containing a partial phage lambda pL sequence (Adams and Galluppi, 1986) containing BamHI sticky ends:

```
5'-GATCCTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACATCG-3'
    3'-GATAGAGACCGCCACAACTGTATTTATGGTGACCGCCACTATGACTCGTGTAGCCTAG-5'
```

The resulting plasmid pMON6001 carries two copies of the synthetic phage lambda pL promoter fragments as direct repeats in the BamHI site of pMON25 in the correct orientation to promote transcription of the *E. coli* EPSP synthase coding sequence.

Plasmid pMON6001 was cleaved with NcoI and EcoRI and the 3 kb fragment isolated from an agarose gel. This fragment was mixed with the small 100 bp NcoI-EcoRI fragment purified from M8O:9. Following ligation and transformation a clone was identified that contained the small 100 bp NcoI-EcoRI fragment corresponding to the 5′ end of the "mature" EPSP synthase of petunia. This construct was designated pMON9544.

Plasmid pMON9544 was digested with EcoRI and treated with alkaline phosphatase. The EcoRI fragment of pMON9544 was mixed with pMON9556 DNA that had been cleaved with EcoRI to release a 1.4 kb fragment encoding the 3′ portion of the petunia EPSP synthase coding sequence. Following ligation and transformation, a clone was identified that could complement an *E. coli* aroA mutation and carried the 1.4 kb fragment of pMON9556 to give an intact mature coding sequence for petunia EPSP synthase. This plasmid was designated pMON342.

The EcoRI site at the 3′ end of the EPSP synthase in pMON342 was replaced with a ClaI site to facilitate construction. This was accomplished by partial digestion with EcoRI followed by digestion with mungbean nuclease to make the ends blunt. ClaI linkers (5′-CATCGATG-3′, New England Biolabs) were added to the blunt ends by ligation with T4 DNA ligase. The mixture was digested with ClaI to produce sticky ends, and the 5 kb EcoRI partial digest was isolated from an agarose gel and ligated with T4 DNA ligase. This plasmid was designated pMON9563.

A 29-nucleotide mutagenic deoxyoligonucleotide having the following sequence:
5′-GCCGCATTGCTGTAGCTGCATTTCCAAGG-3′
was synthesized for introducing the alanine for glycine substitution at position 101 using an automated DNA synthesizer (Applied Biosystems, Inc.). The deoxyoligonucleotide was purified by preparative polyacrylamide gel electrophoresis.

The 660 bp EcoRI-HindIII fragment of pMON9563 was subcloned into a EcoRI-HindIII digested M13mp10 bacteriophage vector (New England Biolabs). The single-stranded template DNA was prepared from the subclone as described in the M13 cloning and sequencing handbook by Amersham, Inc. (Arlington Heights, IL) and oligonucleotide mutagenesis reactions were performed as described by Zoller and Smith (1983) using the oligonucleotide described above. This plasmid was designated M9551: The 660 bp EcoRI-HindIII fragment of M9551 was inserted into pMON9563 between the EcoRI and HindIII sites, replacing the corresponding wild type fragment. This plasmid was designated pMON9566.

The double pL promoter used to express the petunia EPSP synthase in pMON342 and pMON9566 leads to a very high level of expression of the enzyme. The enzyme is present at such high concentration that bacteria harboring either of these plasmids are tolerant to very high levels of glyphosate (>50μM) in growth media, even though the enzyme produced by pMON342 is the wild type form. In order to produce a plasmid that would allow for selection of glyphosate tolerant forms of the enzyme it was necessary to replace the high expressing lambda phage pL promoter with a much weaker promoter sequence. A plasmid for identifying such a promoter was constructed as follows: pMON9544, the precursor to pMON342, was digested with BamHI to remove the pL promoters, and was recircularized by ligation resulting in pMON362. The EcoRI fragment of pMON9556 containing the 3′-portion of the petunia EPSPS cDNA was then inserted into the EcoRI site of this plasmid reconstituting the entire coding sequence. The resulting plasmid, pMON364 is identical to pMON342 except that there is no promoter 5′ of the EPSP synthase coding sequence.

To facilitate future cloning steps, the EcoRI/PstI fragment of pMON364 from which the promoter elements had been deleted was ligated to the EcoRI/PstI fragment of pMON9563 containing most of the EPSP synthase cDNA creating pMON9564. This plasmid is identical to pMON364 except that it has a unique ClaI site at the 3′-end of the EPSP synthase cDNA and a unique EcoRI site within the EPSP synthase coding sequence. Transformation of a aroA *E. coli*, such as SR481 (Bachman et al., 1980; Padgette et al., 1987) failed to complement the mutation, thus demonstrating the effective deletion of the promoter region and the inability of this plasmid to produce EPSP synthase in *E. coli*. An empirical

screening approach was used to isolate promoters with an appropriate low level expression in *E. coli* as follows.

B) Generation of a series of promoter constructs.

Chromosomal DNA isolated from the *E. coli* strain SR20 (GM42 hfr, his-, dam3-) was digested completely with MboI. The MboI fragments were cloned into the BglII site of plasmid pMON9564. The BglII site is in a multilinker located upstream of the promoterless petunia EPSPS coding sequence which had been tailored for expression in *E. coli*. The ligation mixture was used to transform *E. coli* strain SR481, the aroA- variant lacking endogenous EPSPS activity. Forty-one colonies were obtained which contained MboI fragments with sufficient promoter activity to express the Petunia EPSPS cDNA, complementing the aroA defect in SR481 and supporting cell growth on minimal medium. The 41 colonies were streaked individually onto MOPS minimal medium containing glyphosate at 1, 5, 10, 15 and 20μM concentrations. The amount of cell growth on increasing concentrations of glyphosate was used as a measure of the relative promoter strength of each MboI fragment expressing the petunia EPSPS coding sequence. To further characterize each of the MboI promoter fragments, plasmid miniprep DNA was prepared by the alkaline lysis method from each of the 41 colonies and was digested individually with EcoRI, BamHI, HindIII, ClaI and NcoI. Those restriction enzymes were chosen because they cut within, or flank the petunia EPSPS coding sequence and would be used for mobilizing mutagenized fragments. Therefore, ideal promoter fragments would not contain restriction sites for any of those enzymes. There were 8 MboI fragments with varying degrees of promoter activity which lacked restriction sites for the enzymes listed above. Two of them, pMON8105 and pMON8143, were selected for further characterization. Both plasmids complemented the aroA defect and supported the growth of SR481 on minimal medium containing up to 1mM (pMON8105) and 20mM (pMON8143) glyphosate.

C) Testing the Expression Vectors.

The heterologous expression system was tested with a known variant to see if glyphosate resistant variants of petunia EPSPS could be selected. The glyphosate resistant mutation, glycine (101) to alanine, was introduced into the petunia EPSPS coding sequence of both pMON8105 and pMON8143 expression vectors to generate pMON8135 and pMON8152, respectively. That was achieved by replacing the 660 bp EcoRI-HindIII region from both vectors with the 660bp EcoRI-HindIII region from pMON9566 which contained the glycine (101) to alanine mutation. Both pMON8135 and pMON8152 were used to transform SR481. The pMON8152 construct was able to complement the aroA defect in SR481 and support cell growth on minimal medium containing up to 50mM glyphosate.

The weakly expressing pMON8135 (Figure 2) construct containing the variant enzyme sequence was not able to complement the aroA defect in SR481 and did not support cell growth on minimal medium. A culture of SR481 cells carrying the pMON8135 plasmid was assayed to demonstrate that the petunia EPSP synthase enzyme was expressed. Plasmid pMON8135 has a specific activity of 41 nmol/min/mg protein and pMON8105 has a specific activity of 28 nmol/min/mg protein. So, the pMON8135 construct was expressed in *E. coli* with a specific activity similar to its parental construct pMON8105. It was then hypothesized that the elevated K_m for PEP of the variant enzyme (198 μM versus 5.2 μM for the wild type) resulted in a relatively inefficient EPSP synthase enzyme that was unable to complement the aroA mutation when the enzyme was produced at this low level. This result demonstrated the importance of the K_m for PEP and the ability of a variant petunia EPSPS enzyme to complement aroA when weakly expressed in *E. coli*. If a variant enzyme has a high K_m for PEP, then a greater level of expression is required to complement aroA. The weakly expressing vector, pMON8105, therefore, provides a novel, powerful selection tool for identifying petunia EPSPS variants which have relatively low K_m values for PEP. In combination with glyphosate selection, variants which combine significant glyphosate tolerance with low K_m values for PEP can be obtained. This implies that not only can new variants of the wild type enzyme be obtained from this system, but it can also be used to select for second site mutations in the glycine (101) to alanine variant coding sequence that lower the K_m for PEP while maintaining glyphosate tolerance. This unexpectedly powerful selection system constitutes one part of the present invention. Those skilled in the art will recognize that one can use other strains of aroA bacteria, other methods of insertion, other sources of random DNA fragments and coding sequences from organisms other than petunia while not departing from the spirit and scope of the invention.

D) In vivo Mutagenesis of pMON8135 with Ethyl Methane Sulfonate.

The following mutagenesis procedure serves as an example of the application of this selection system for obtaining such second site variants of the glycine (101) to alanine variant of the petunia enzyme. Ethyl methane sulfonate (EMS) is a chemical mutagen commonly used in bacterial genetics, but it requires growing the bacterial cultures in minimal medium. Since pMON8135 does not complement aroA in SR481, a prototrophic strain of *E. coli* had to be employed.

pMON8135 was transformed into the *E. coli* strain JM109. A 3ml culture was grown to saturation overnight at 37C in 2XYT media containing 50µg/ml carbenicillin. A 0.5 ml aliquote of the saturated culture was diluted 40 fold into 20ml 2XYT medium in a side arm flask. The diluted culture was shaken continuously in a water bath at 37C and the growth was monitored using a Klett-Summerson photoelectric colorimeter until a Klett value of 145 was reached. The culture was then mixed with an equal volume (20ml) of an EMS stock solution which contained 0.8ml EMS (Sigma Chemical, St. Louis, Mo.) and 19.2ml 1X MOPS minimal medium. After being shaken for 2 hours at 37C, the 40ml culture was diluted 10 fold with 1X MOPS media to a final volume of 400ml. The diluted culture was then shaken for 3 hours at 37C. The cells were pelleted in a 500ml plastic centrifuge bottle using a Beckman JA10 rotor for 10 min at 7000 rpm and at 5C. The cells were then resuspended and washed in 100ml of 1X MOPS media and then pelleted as above. The bacterial cell pellet was resuspended in 200ml of 2XYT growth medium and shaken in a 1 liter flask for 90 minutes at 37C. The cells were then pelleted again as above and were frozen at -20C. The pellet was thawed and the mutagenized pMON8135 plasmid DNA was then extracted following a standard alkaline lysis procedure.

E) Screening for Glyphosate Resistant Coding Sequence Variants.

A multi-step screening procedure was used to identify glyphosate resistant variants of petunia EPSPS. The first screening step involved the transformation of *E. coli* with the EMS mutagenized pMON8135 plasmid DNA and selecting for glyphosate resistant colonies on minimal medium containing glyphosate. The SR481 aroA *E. coli* strain had a very low transformation frequency, yielding at best 10^4 transformants per µg of plasmid DNA. To overcome that problem, the *E. coli* strain JM101 was used because transformation efficiencies of up to 10^8 transformants per µg of plasmid DNA could be routinely obtained. However, JM101 contained a fully functional aroA gene and was able to grow on MOPS minimal medium. By plating JM101 on minimal medium plates containing increasing concentrations of glyphosate, it was determined that 2mM glyphosate would inhibit the endogenous EPSP synthase enzyme activity and growth of JM101 on minimal media. Since the weakly expressing wild type petunia EPSP synthase cDNA construct (pMON8105) could not support the growth of the aroA-*E. coli* strain, SR481, on 1mM glyphosate and the corresponding glycine 101 to alanine construct (pMON8135) could not complement the bacterial aroA, then a prototrophic strain of *E. coli* can be used for the selections if the glyphosate concentrations are greater than 2mM. The EMS mutagenized pMON8135 plasmid DNA was transformed into JM101 and glyphosate resistant variants were selected on MOPS minimal medium containing 5mM glyphosate.

The glyphosate resistant colonies contained pMON8135 plasmids with a variety of mutations, including promoter mutations, copy number mutations and glyphosate resistant mutations in the petunia EPSP synthase coding sequence. Mutations that increased plasmid copy number or increased the strength of the promoter that was used to drive the EPSP synthase gene would increase the amount of EPSP synthase enzyme in the bacteria and would confer an aroA positive glyphosate tolerant phenotype to the cells. To eliminate mutations in the non-coding regions of the EMS mutagenized pMON8135 plasmid, the glyphosate resistant colonies were pooled together into 2XYT liquid media containing 50µg/ml carbenicillin and grown overnight at 37C with agitation to aerate the cells. The cells were then pelleted from the saturated cultures by centrifugation and the plasmid DNA was extracted using the alkaline lysis procedure. The plasmid DNA was then digested completely with EcoRI and ClaI enzymes and the 1.6kb petunia EPSPS coding sequence region was then purified out of a 0.8% SeaPlaque (FMC Corporation) low gelling temperature agarose gel. The 1.6kb EcoRI-ClaI fragment was used to replace the analogous fragment containing the wild-type coding sequence in the non-mutagenized pMON8105 expression vector by ligating it to the 3.83kb EcoRI-ClaI vector fragment of this plasmid which had been isolated as above. The ligation mixture was then used to transform JM101 cells, which were plated onto MOPS minimal medium containing 5mM glyphosate to select for glyphosate resistant mutations in the petunia EPSP synthase coding sequence region.

The glyphosate resistant colonies obtained from the transformations of the sub-cloned coding region were further characterized by measuring the rate of growth of each variant in liquid culture in varying

concentrations of glyphosate. This growth curve analysis functioned as a tertiary screen and was performed in the following way:

Glyphosate resistant colonies were picked off the selection plates and inoculated individually into precultures containing 1ml of MOPS medium and 50μg/ml carbenicillin. The precultures were then grown to saturation by shaking the cultures overnight at 37C. The next morning the density of each culture was determined by withdrawing a 100μl aliquote from each and diluting it 10 fold with the addition of 900μl MOPS medium, then reading the optical density at a wavelength of 660nm in a spectrophotometer. The saturated precultures were then diluted to 1% by adding 50μl from each saturated preculture to 5ml of MOPS media containing 0, 5 or 10mM glyphosate. The diluted precultures were grown in glass culture tubes fitted with stainless steel closures, rotating on a wheel at 37C. The glass culture tubes were designed for direct reading in a Klett-Summerson photoelectric colorimeter, which was used to monitor the growth of the bacterial cultures at approximately 3 hour intervals.

One culture, #215, was identified which grew faster than all of the other glyphosate resistant cultures and control cultures in MOPS medium containing 10mM glyphosate. It was the only culture that had grown to saturation within 11 hours of growth in this concentration of glyphosate. The control cultures were pMON8143 and pMON8152 (both described above) in the JM101 *E. coli* host.

F) Characterization of the Glyphosate Resistant Coding Sequence Variants.

The balance of the #215 preculture (~750μl) was used to inoculate 2ml of MOPS medium containing 50μg/ml carbenicillin and was shaken overnight at 37C to reach saturation. Plasmid DNA was isolated from an aliquot of the saturated culture using an alkaline lysis procedure. The plasmid was designated pMON8186. An aliquot of the pMON8186 plasmid was used to transform the E. coli host SR481 (described above) and reselected on MOPS medium containing 10mM glyphosate and 50μg/ml carbenicillin. A single glyphosate resistant colony of pMON8186 was picked off the selection plate and used to inoculate 3ml of 2XYT bacterial medium containing 50μg/ml carbenicillin. The culture was then aerated on rotating wheel at 37C until saturated, then it was used to inoculate a larger 500ml culture. The large culture was grown to saturation by shaking it overnight at 37° C in a water bath. The bacterial cells were lysed and the extracts were assayed for EPSPS activity.

Specifically, the bacterial cell paste was washed twice with 0.9% saline, suspended in buffer (100 mM Tris-HCl, 5 mM benzamidine HCl) and passed twice through the French Pressure Cell at 70.3 kg/sq.cm (1000 psi). The cell extract was separated from the cells by centrifuging at 15,000 x gravity for 10 mins. at 5° C. It was desalted using Sephadex G-50 (Pharmacia, Piscataway, New Jersey). The desalted extract was assayed for EPSP synthase activity as follows:

To an assay mix (40μl) containing shikimate-3-phosphate (2mM), ^{14}C-phosphoenolpyruvate (1mM; 1.2mCi/mmol), ammonium molybdate (0.1 mM), potassium fluoride (5mM) in 50 mM HEPES-KOH, pH 7, was added 10μl of the extract and incubated at 25° C for 2 mins. The reaction was quenched by the addition of 50 μl of 90% ethanol/0.1 M acetic acid, pH 4.5. 70 μl of the reaction mixture was loaded on a SynchroPak AX100 HPLC column (0.4 x 25 cm) and the column was eluted with 0.5 M potassium phosphate, pH 5.5 at 1 ml/min. The radioactivity of the eluent was monitored using a Radiomatic Flo-One Beta Instrument. (Radiomatics, Florida). The EPSP synthase activity was determined by measurement of the conversion of ^{14}C-PEP to ^{14}C-EPSP synthase, both of which are resolved under the above conditions of chromatography. The protein content of the extract was determined by the method of Bradford (Biorad Labs, California). The specific activity of the extract is expressed as nanomoles of EPSP synthase formed/min/mg protein.

Kinetic constants (appKm PEP and appKi glyphosate) were determined for EPSP synthase as described below. Substrate kinetic parameters were determined at pH 7.0 in 50 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer in the presence of 2 mM S3P and varying amounts of ^{14}C-PEP (10μM-400μM), for 2.0 minutes at 25° C. Reactions were quenched with 100mM Na Acetate in ethanol, pH 4.5, centrifuged and analyzed for product EPSP formation by HPLC with flow radioactivity detection. HPLC conditions were 0.35 MKPi, pH 6.5, on a Synchropak AX100 column at 1.0 ml/min. The resulting rates were plotted by hyperbolic plot, Lineweaver-Burk plot and Eadie-Hofstee plot and an average K_m for PEP value obtained. The appKi for glyphosate versus PEP was determined as described for the substrate kinetic constant except in the presence of varying concentrations of glyphosate (0,100,200,400 μM). Initial rate data was plotted as 1/[PEP] versus 1/V and the slopes of the resulting lines were replotted versus [glyphosate].

The assay results showed that the bacterial cells containing the pMON8186 plasmid had an EPSP

synthase activity of 28nmoles of EPSP formed/min/mg of protein. The enzyme was highly resistant to glyphosate as indicated by a K_i for glyphosate of 348μM. The K_m for PEP was determined to be 40μM. The petunia EPSPS glycine (101) to alanine variant has a K_i for glyphosate of 2000μM and a K_m for PEP of 200μM. The K_i/K_m ratio for the pMON8186 encoded glyphosate resistant variant enzyme is 7.7, which is similar to that of the progenitor glycine (101) to alanine variant whose ratio is 10.0. However, the pMON8186 enzyme has a K_m for PEP that is more than four fold lower than the glycine (101) to alanine variant. The lowering of the K_m for PEP makes the pMON8186 variant enzyme more efficient kinetically, as demonstrated by its ability to support the growth of *E. coli* in MOPS medium containing high concentrations of glyphosate. This demonstrated that our selection system allowed for the induction and identification of mutations of the petunia EPSPS glycine (101) to alanine variant enzyme which would maintain the glyphosate resistant properties of the original variant, but lower the K_m for PEP. The pMON8186 results also demonstrated that the improvements in the K_m for PEP could be selected in the heterologous bacterial expression system described above. A variant petunia EPSP synthase containing the glycine (101) to alanine substitution and the glycine (144) to asparagine substitution exhibited a K_m for PEP of 91μM and a K_i for glyphosate of 960μM (K_i/K_m = 10.5).

G) Identification of the pMON8186 Mutation.

To identify the EMS induced mutation responsible for the improved glyphosate resistant properties of the pMON8186 variant enzyme, it was first localized within the coding sequence region. This was achieved by subcloning the 5′ and 3′ halves individually into a bacterial overexpression vector and determining the kinetic properties of each subclone as follows: The 660bp EcoRI-HindIII fragment from the petunia EPSPS coding sequence in pMON9767 was replaced with the analogous EcoRI-HindIII fragment from pMON8186. Plasmid pMON9767 is a derivative of pMON9566 (described above) in which an XbaI site had been created at the 3′ end of the petunia EPSPS glycine (101) to alanine variant coding sequence by site directed mutagenesis. Similarly, the 940bp 3′ HindIII-ClaI fragment of the petunia EPSPS coding sequence region from pMON9767 was replaced with the analogous HindIII-ClaI fragment from pMON8186. The constructs were then transformed into SR481 and plated on MOPS medium containing 50μg/ml carbenicillin. Large scale cultures (50ml 2XYT containing 50μg/ml carbenicillin) of each subclone were prepared from single colonies picked off the selection plates. The cultures were grown to saturation by shaking them overnight in a 37C waterbath. The cells were pelleted and lysed. The bacterial extracts were assayed as described above and the approximate K_i and K_m values were determined for each pMON8186 subclone. The kinetic values for the EcoRI-HindIII region subclone were similar to those of the intact pMON8186, while those of the HindIII-ClaI subclone were similar to those of pMON8135. Thus, the EMS induced, second site mutation responsible for the excellent kinetic properties of pMON8186 was located on the 660bp EcoRI-HindIII fragment. That subclone of the EcoRI-HindIII region was designated pMON8191.

The 660bp EcoRI-HindIII fragment of pMON8186 was sequenced to determine the exact nucleotide change and the corresponding amino acid change responsible for the new kinetic properties of the pMON8186 encoded variant enzyme. The 660bp EcoRI-HindIII fragment from pMON8186 was inserted into EcoRI-HindIII cut M13mp18 and M13mp19 bacteriophage vector DNAs and were designated M8059 and M8058, respectively. Following transformations into JM101, single plaques were picked and single strand template DNA was prepared from each (protocol from Amersham, M13 cloning and sequencing handbook). The template DNAs were sequenced by the method of Sanger using the reagents and protocol from a commercially available DNA sequencing kit from United States Biochemical Corporation. The presence of the glycine (101) to alanine substitution was confirmed in the DNA sequence. In addition, there was a single guanine to adenine transition at the second nucleotide position of the GGT codon for glycine (144) in the mature petunia EPSP synthase, resulting in a glycine to aspartate amino acid substitution at the 144 position. The guanine to adenine transition is consistent with the type of mutation known to be induced by EMS. Thus, the improved kinetic properties of the pMON8186 encoded glyphosate resistant petunia EPSPS variant enzyme are due to a combination of two substitutions: one resulting in the glycine (101) to alanine change, the other resulting in a glycine (144) to aspartic acid amino acid change.

The petunia EPSP synthase coding sequence containing the glycine (101) to alanine and glycine (144) to aspartic acid substitutions was engineered for appropriate expression in plant cells. Construction of the intermediate plant transformation vector and *Agrobacterium tumefaciens*-based transformations of plant cells is described below.

Construction of pMON915

The plant transformation vector pMON915 was derived from the pMON895 vector. The pMON895 plasmid (Figure 3) is made up of the following segments of DNA. The first segment is a 0.93 Kb AvaI to engineered EcoRV fragment isolated from transposon Tn7 that encodes bacterial spectinomycin/streptomicin resistance (Spc/Str), which is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*. This is joined to the 1.61 Kb segment of DNA encoding a chimeric kanamycin resistance gene which permits selection of transformed plant cells. The chimeric gene (P-35S/KAN/NOS 3′) consists of the cauliflower mosaic virus (CaMV) 35S promoter, the neomycin phosphotransferase type II (KAN) gene, and the 3′-nontranslated and flanking regions of the nopaline synthase gene (NOS 3′). The next segment is the 0.75 Kb ori-V containing the origin of replication from the RK2 plasmid. It is joined to the 3.1 Kb SalI to PvuI segment of pBR322 (ori-322) which provides the origin of replication for maintenance in *E. coli* and the bom site for the conjugational transfer into the *Agrobacterium tumefaciens* cells. The ori-V and ori-322 segments also provide homology for the recombination of the vector into the disarmed pTiT37-CO plasmid to form a hybrid T-DNA as described below. The next segment is the 0.36 Kb PvuI to BclI from pTiT37 that carries the nopaline-type T-DNA right border.

The pMON895 plasmid contains next the 3.14 Kb DNA segment that encodes a chimeric gene for expression of the petunia 5-enolpyruvylshikimate-3-phosphate synthase. The chimeric gene consists of the 0.66 Kb CaMV 35S promoter enhanced as described by Kay et al. (1987) (P-e35S), followed by the 2 Kb coding sequence for the petunia EPSPS with the glycine (101) to alanine substitution (PrePEPSPS:2), and the 0.48 Kb 3′ nontranslated region of the soybean alpha′ subunit of the beta-conglycinin gene (7S 3′) (Schuler et al. 1982).

Plasmid pMON915 (Figure 4) was constructed from three DNA fragments:

1. The 7.91 Kb BglII to XbaI fragment from pMON895 containing the P-e35S promoter, the Spc/Str gene, P-35/KAN/NOS 3′, ori-V, ori-PBR, and the right border.
2. The 1.27 Kb XbaI to EcoRI fragment from pMON8191 containing the petunia EPSP synthase gene with the glycine (101) to alanine and glycine (144) to aspartic acid substitutions.
3. The 0.31 Kb EcoRI to BglII fragment from pMON895, which contains the petunia EPSP synthase chloroplast transit peptide.

The pMON915 plasmid was introduced into the ACO *Agrobacterium* strain. The strain carries the disarmed pTiT37-CO nopaline type plasmid. Referring to Figure 5, a restriction map is provided of the T-DNA region of the A208 *Agrobacterium tumefaciens* strain pTiT37 plasmid, which was disarmed to create the ACO strain. The hatched boxes show the segments of the Ti plasmid DNA which were used to provide homology for recombination and replacement of the T-DNA. The T-DNA segment was replaced by the Tn601 bacterial kanamycin resistance gene (KnR) segment joined to the oriV and pBR322 segment homologous to the vectors described above. The recombination between the disarmed pTiT37-CO and pMON915 plasmid takes place through the pBR322 oriV area of homology, resulting in the hybrid T-DNA which contains the entire pMON915 DNA. On cultivation of the *Agrobacterium* with plant cells, the hybrid T-DNA segment between the left and right borders is transferred to the cells and integrated into the genomic DNA.

The variant EPSP synthase polypeptides of the present invention may be prepared by either polypeptide synthesis or isolation and mutagenesis of a EPSP synthase gene to produce the above described glyphosate-tolerant molecule. Since it is foreseen that the greatest utility of the present invention is in the preparation of glyphosate-tolerant plants, nucleotide sequences (either cDNA or genomic) encoding the glyphosate-tolerant EPSP synthase can be easily prepared in the following manner.

cDNA Coding Sequences

Total RNA is isolated from the source material which includes, but is not necessarily limited to, fungi and plant tissue. PolyA-mRNA is selected by oligodT cellulose chromatography. A cDNA library is then prepared using the polyA-mRNA. The cDNA library is then screened using a previously cloned EPSP synthase sequence or a suitable oligonucleotide probe. Suitable oligonucleotide probes include probes based on the conserved region having the amino acid sequence (L-G-N-A-G-T-A) or probes based on the amino acid sequence of other portions of the EPSP synthase molecule. The cDNA fragments selected by hybridization are then sequenced to confirm that the fragment encodes the EPSP synthase and to determine the DNA sequence encoding and adjacent to the conserved amino acid sequence described above.

The EPSP synthase clone is then altered by oligonucleotide mutagenesis to insert the DNA substitution necessary to result in the alanine for glycine substitution in the first conserved amino acid sequence and an aspartic acid for glycine substitution in a second conserved amino acid sequence as previously described. The above procedure produces a cDNA sequence which encodes the glyphosate-tolerant EPSP synthase of the present invention based on the wild-type EPSP synthase of the selected source material. This structural coding sequence can be inserted into functional chimeric gene constructs and inserted into suitable plant transformation vectors to be used in preparing transformed plant cells and regenerated plants using the methodology described herein.

Genomic EPSP Synthase Clone

Generally it is preferred that the plant tissue from the plant species to be transformed also serve as the source material for the DNA coding sequence for the glyphosate-tolerant EPSP synthase of the present invention. In this way, one would easily obtain the chloroplast transit peptide coding sequence from the plant species to be transformed. In some cases, it may be beneficial to utilize a genomic clone from the plant species which comprises the introns normally found in the endogenous EPSP synthase gene. The general method described above is also applicable with the exception that the probes are used to screen a genomic DNA library constructed from the selected plant tissue. Detailed examples better elucidating this preparation of cDNA and genomic DNA glyphosate-tolerant EPSP synthase constructs of the present invention are provided below.

PREPARATION OF EPSP SYNTHASE PLANT TRANSFORMATION VECTORS

I. cDNA ENCODING THE EPSP SYNTHASE OF PETUNIA

Described below is the methodology employed to prepare the cDNA clone of petunia EPSP synthase which was used in the mutagenesis procedure described above. Clones of wild-type EPSP synthases from other plant sources can be obtained in a similar manner and the above described mutations introduced by site directed mutagenesis.

A. Creation of MP4-G Cell Line

The starting cell line, designated as the MP4 line, was derived from a Mitchell diploid petunia (see e.g., Ausubel 1980). The MP4 cells were suspended in Murashige and Skoog (MS) culture media, (GIBCO, Grand Island, N.Y.) All transfers involved dispensing 10 ml of suspension cultures into 50 ml of fresh media. Cultivation periods until the next transfer ranged from 10 to 14 days, and were based on visual indications that the culture was approaching saturation.

Approximately 10 ml of saturated suspension culture (containing about x 10^6 cells) were transferred into 50 ml of MS media containing 0.5 mM glyphosate. The sodium salt of glyphosate was used throughout the experiments described herein. The large majority of cells were unable to reproduce in the presence of the glyphosate. The cells which survived (estimated to be less than 1% of the starting population) were cultured in 0.5 mM glyphosate and transferred to fresh media containing glyphosate every 10 to 14 days.

After two transfers, the surviving cells were transferred into fresh media containing 1.0 mM glyphosate. After two transfers at 1.0 mM, the surviving cells were transferred sequentially into 2.5 mM glyphosate, 5.0 mM glyphosate, and 10 mM glyphosate.

The MP4-G cells prepared as described above were subsequently shown by a Southern blot analysis (Southern, 1975) to have about 15-20 copies of the EPSP synthase gene, due to a genetic process called "gene amplification" (see e.g. Schimke 1982). Although spontaneous mutations might have occurred during the replication of any cell, there is no indication that any mutation or other modification of the EPSP synthase gene occurred during the gene amplification process. The only known difference between the MP4 and the MP4-G cells is that the MP4-G cells contain multiple copies of an EPSP synthase gene and possibly other genes located near it on the chromosomes of the cells.

B. Purification and Sequencing of EPSP Synthase Enzymes

Petunia cells from the MP4-G cell line were harvested by vacuum filtration, frozen under liquid N_2, and ground to a powder in a Waring blender. The powder was suspended in 0.2 M Tris-HCl, pH 7.8, containing 1 mM EDTA and 7.5% w/v plyvinylpolypyrrolidone. The suspension was centrifuged at about 20,000 x gravity for 10 min to remove cell debris. Nucleic acids were precipitated from the supernatant by addition of 0.1 volume of 1.4% protamine sulfate and discarded.

The crude protein suspension was purified by five sequential steps (see Mousdale 1984 and Steinrucken 1985) which involved: (1) ammonium sulfate precipitation; (2) diethylaminoethyl cellulose ion exchange chromatography; (3) hydroxyapatite chromatography; (4) hydrophobic chromatography on a phenylagarose gel; and (5) sizing on a Sephacryl S-200 gel.

The purified EPSP synthase polypeptide was degraded into a series of individual amino acids by Edman degradation by a Model 470A Protein Sequencer (Applied Biosystems Inc., Foster City, CA), using the methods described in Hunkapiller 1983a. Each amino acid derivative was analyzed by reverse phase high performance liquid chromatography, as described by Hunkapiller 1983b, using a cyanopropyl column with over 22,000 theoretical plates (IBM Instruments, Wallingford CT). A partial amino acid sequence for petunia EPSP synthase is shown in Table 1.

Table 1

Petunia EPSP Synthase Sequences						
	8	9	10	11	12	13
Amino Acid:	Gln	Pro	Ile	Lys	Glu	Ile
mRNA strand:	5′-CAP	CCN	AUU C A	GAP	CAP	AUU C A
Complementary DNA strand:	3′-GTQ	GGN	TAA G U	TTQ	CTQ	TAA G U
Synthetic DNA Probes:						
EPSP1:	3′-GTQ	GGP	TAP	TTQ	CTQ	TA
EPSP2:	3′-GTQ	GGQ	TAP	TTQ	CTQ	TA
EPSP3:	3′-GTQ	GGN	TAT	TTQ	CTQ	TA
Exact mRNA Sequence:	5′-CAA	CCC	AUU	AAA	GAG	AUU

C. Synthasis of Probes

Using the genetic code, the amino acid sequence indicated in Table 1 was used to determine the possible DNA codons which are capable of coding for each indicated amino acid. Using this information, three different probe mixtures were created and designated as EPSP-1, EPSP-2, and EPSP-3, as shown in Table 1. In this table, A, T, U, C, and G represent the nucleotide bases: adenine, thymine, uracil, cytosine and guanine. The letters P, Q, and N are variables; N represents any of the bases; P represents purines (A or G); Q represents pyrimidines (U, T, or C).

All oligonucleotides were synthesized by the method of Adams 1983. Whenever an indeterminate nucleotide position (P, Q or N) was reached, a mixture of appropriate nucleotides was added to the reaction mixture. Probes were labeled 20 pmol at a time shortly before use with 100 μCi γ-[^{32}P]-ATP (Amersham) and 10 units polynucleotide kinase in 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, and 0.1 mM spermidine. After incubation for 1 hr. at 37° C, the probes were repurified on either a 20% acrylamide, 8 M urea gel or by passage over a 5 ml column of Sephadex G25 in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.

D. Preparation of mRNA and Preliminary Testing of Probes

(a) Poly-A mRNA

Total RNA was isolated from the MP4 (glyphosate sensitive) and MP4-G (glyphosate resistant) cell lines as described by Goldberg 1981. Total RNA was further sedimented through a CsCl cushion as described by Depicker 1982. Poly-A mRNA was selected by oligo-dT cellulose chromatography. The yield of poly-A RNA was 1.1 micrograms (µg) per gram of MP4 cells and 2.5 µg/gm of MP4-G cells.

(b) Gel Processing of RNA

Ten µg of poly-A RNA from the MP4 or MP4-G cell lines were precipitated with ethanol and resuspended in 1 x MOPS buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate and 1 mM EDTA, ph 8.0) containing 50% formamide and 2.2 M formaldehyde. RNA was denatured by heating at 65 °C for 10 min. One-fifth volume of a loading buffer containing 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol was then added. RNA was fractionated on a 1.3% agarose gel containing 1.1 M formaldehyde until bromophenol blue was near the bottom. HaeIII-digested ϕX174 DNA, labelled with ^{32}P, was run as a size standard. The DNA markers indicated approximate sizes for the RNA bands.

(c) Transfer of RNA to Nitrocellulose

RNA was transferred to nitrocellulose (#BA85, Schleicher & Schuell, Keene, NH) by blotting the gels overnight using 20X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) as the transfer buffer. After transfer, filters were air-dried and baked in a vacuum oven for 2-3 hrs at 80 °C.

(d) Preliminary Hybridization with Radioactive Probes

Filters were prehybridized in 6 x SSC, 10 x Denhardt's solution (1 x Denhardt's solution is 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.5% NP-40, and 200 µg/ml *E. coli* transfer RNA at 50 °C for 4 hrs. Hybridization was carried out in a similar fresh solution containing 2 x 10^5 cpm/ml of either EPSP-1 or EPSP-2 probe for 48 hrs at 32 °C. The EPSP-3 probe was not tested since it contained a codon (ATA) that is rarely found in the petunia genome. Hybridization temperature (32 °C) used in each case was 10 °C below the dissociation temperature (Td) calculated for the oligonucleotide with the lowest GC content in a mixture. The Td of the probe was approximated by the formulate 2 °C x (A + T) + 4 °C x (G + C).

(e) Filter Washing

The filters were washed twice for 15-20 min at room temperature in 6 x SSC and then for 5 min at 37 °C with gentle shaking. Filters were then wrapped in plastic film and autoradiographed for 12-14 hrs at -70 °C with two intensifying screens. The filters were then washed again for 5 min with gentle shaking at a temperature of 42 °C. The filters were autoradiographed again for 12-14 hrs. The autoradiographs indicated that the probe EPSP-1 hybridized to an RNA of approximately 1.9 kb in the lane containing the poly-A RNA from the MP4-G cell line. No hybridization to this RNA was detected in the lane containing the poly-A RNA from the MP4 cell line. This result was attributed to overproduction of EPSP synthase mRNA by the MP4-G cell line. The probe EPSP-2, which differs from EPSP-1 by a single nucleotide, showed barely detectable hybridization to the 1.9 kb mRNA of the MP4-G cell line but hybridized strongly to a 1.0 kb mRNA from both cell lines. However, the 1.0 kb DNA was not sufficient to encode a polypeptide of 50,000 daltons, and it is believed that one of the sequences in the EPSP-2 probe hybridized to an entirely different sequence in the library. These results suggested that degenerate probe mixture EPSP-1 contained the correct sequence for EPSP synthase. This mixture was used in all subsequent degenerate probe hybridization experiments.

E. Preparation of λgt 10 cDNA library

(a) Materials Used

AMV reverse transcriptase was purchased from Seikagaku America, Inc., St. Petersburg, Florida; the large fragment of DNA polymerase I (Klenow polymerase) was from New England Nuclear, Boston, MA; S1 nuclease and tRNA were from Sigma; AcA 34 column bed resin was from LKB, Gaithersburg, MD; EcoRI, EcoRI methylase and EcoRI linkers were from New England Biolabs, Beverly MA; RNAsin (ribonuclease inhibitor) was from Promega Biotech, Madison, Wisc. and all radioactive compounds were from Amersham, Arlington, Hts:, IL.

The λgt10 vector (ATCC No. 40179) and associated *E. coli* cell lines were supplied by Thanh Huynh and Ronald Davis at Stanford University Medical School (see Huynh 1985). This vector has three important characteristics: (1) it has a unique EcoRI insertion site, which avoids the need to remove a center portion of DNA from the phage DNA before inserting new DNA; (2) DNA ranging in size from zero to about 8,000 bases can be cloned using this vector; and, (3) a library can be processed using *E. coli* MA150 cells (ATCC No. 53104) to remove clones which do not have DNA inserts.

(b) cDNA First Strand Synthesis

Poly-A mRNA was prepared as described in section D.(a) above, and resuspended in 50 mM Tris (pH 8.5), 10 mM $MgCl_2$, 4 mM DTT, 40 mM KCl, 500 μM of d(AGCT)TP, 10 μg/ml dT_{12-18} primer, and 27.5 units/ml RNAsin. In a 120 μl reaction volume, 70 units reverse transcriptase were added per 5 μg of poly-A RNA. One reaction tube contained γ-^{32}P-dCTP (5 uCi/120 μl reaction) to allow monitoring of cDNA size and yield and to provide a first strand label to monitor later reactions. In order to disrupt mRNA secondary structure, mRNA in H_2O was incubated at 70° C for 3 min and the tube was chilled on ice. Reverse transcriptase was added and the cDNA synthesis was carried out at 42° C for 60 min. The reaction was terminated by the addition of EDTA to 50 mM. cDNA yield was monitored by TCA precipitations of samples removed at the start of the reaction and after 60 min. Following cDNA synthesis, the cDNA existed as a cDNA-RNA hybrid. The cDNA-RNA hybrid was denatured by heating the mixture in a boiling water bath for 1.5 min, and cooled on ice.

(c) Second Strand DNA Synthesis

Single-stranded cDNA was allowed to self-prime for second strand synthesis. Both Klenow polymerase and reverse transcriptase were used to convert ss cDNA to ds cDNA. Klenow polymerase is employed first since its 3′-5′ exonuclease repair function is believed to be able to digest non-flush DNA ends generated by self-priming and can then extend these flush ends with its polymerase activity. Reverse transcriptase is used in addition to Klenow polymerase, because reverse transcriptase is believed to be less likely to stop prematurely once it has bound to a template strand. The Klenow polymerase reaction was in a final 100 μl volume excluding enzyme. The reaction mix included 50 mM HEPES, pH 6.9, 10 mM $MgCl_2$, 50 mM KCl, 500 μM of each dNTP and cDNA. To begin the reaction, 20 to 40 units of Klenow polymerase (usually less than 5 μl) were added and the tubes incubated at 15° C for 5 hrs. The reaction was terminated by the addition of EDTA to 50 mM. The mix was extracted with phenol and the nucleic acids were precipitated, centrifuged and dried.

The reverse transcriptase reaction to further extend the anti-complementary DNA strand was performed as described for the reaction to originally synthesize cDNA, except dT_{10-18} primer and RNAsin were absent, and 32 units of reverse transcriptase were used in a 120 μl reaction. The reaction was terminated by the addition of EDTA to 50 mM. The mixture was extracted with an equal volume of phenol and the nucleic acid was precipitated, centrifuged and dried.

(d) S1 Nuclease Treatment

200 μl of 2x S1 buffer (1x S1 buffer is 30 mM sodium acetate, pH 4.4, 250 mM NaCl, 1 mM $ZnCl_2$), 175 μl of H_2O and 525 units of S1 nuclease were added to the tubes containing 125 μl of the second

strand synthesis reaction product. The tubes were incubated at 37° C for 30 min and the reaction was terminated by addition of EDTA to 50 mM. The mixture was extracted with an equal volume of phenol/chloroform (1:1). The aqueous phase was extracted with ethyl ether to remove residual phenol. The DNA was precipitated with ethanol and air dried.

(e) EcoRI Methylation Reaction

Since the ds cDNAS were copied from a large variety of mRNAS, many of the ds cDNAS probably contained internal EcoRI restriction sites. It was desired to protect such cleavage sites from EcoRI cleavage, to enable the use of blunt-ended EcoRI linkers which were subsequently cleaved with EcoRI to create cohesive overhangs at the termini.

In an effort to prevent the undesired cleavage of internal EcoRI sites, the ds cDNA was methylated using EcoRI methylase. DNA pellets were dissolved in 40 µl of 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM DTT. Four µl of 100 uM S-adenosyl-L-methionine and 1 µl (80 units) of EcoRI methylase were added. Tubes were incubated at 37° C for 15 min and then at 70° C for 10 minutes to inactivate the methylase.

It was subsequently discovered that the methylation reaction described above was unsuccessful in preventing EcoRI cleavage at an internal site within the EPSP synthase coding region, apparently because of inactive methylase reagent. The cleavage of the internal EcoRI site required additional steps to isolate a full-length cDNA, as described below. To avoid those additional steps, the methylation reagents and reaction conditions should be used simultaneously on the cDNA and on control fragments of DNA, and protection of the control fragments should be confirmed by EcoRI digestion before digestion is performed on the cDNA.

(f) DNA Polymerase I Fill-In Reaction

To the tube containing 45 µl of cDNA (prepared as described above) were added 5 µl of 0.1 M $MgCl_2$, 5 µl of 0.2 mM d(ACGT)TP and 10 units of DNA polymerase I. The tube was incubated at room temperature for 10 min. The reaction was terminated by the addition of EDTA to 25 mM. One microgram of uncut λgt10 DNA was added as a carrier and the mix was extracted with phenol/chloroform (1:1). The nucleic acid in the mix was precipitated with ethanol, centrifuged and dried.

(g) Ligation of EcoRI Linkers to Methylated ds cDNA

Approximately 400 pmoles of EcoRI linkers (5′-CGGAATTCCG-3′) were dissolved in 9 µl of 20 mM Tris, pH 8.0, 10 mM $MgCl_2$, 10 mM DTT containing 50 µCi of γ-^{32}P ATP (5000 Ci/mmole) and 2 units of T4 polynucleotide kinase. The oligonucleotides were incubated at 37° C for 30 minutes to allow them to anneal to each other, creating double-stranded, blunt-ended linkers. 2 units of T4 polynucleotide kinase and 1 µl of 10 mM ATP were added and incubated at 37° C for an additional 30 min. The linkers were stored at -20° C. the methylated DNA pellet was resuspended in tubes containing 400 pmoles of the kinased linkers. Ligation of the EcoRI linkers to the methylated DNA was carried out by adding 1 µl of T4 ligase and incubating the reaction mixture at 12-14° C for 2 days.

(h) Digestion with EcoRI to Create Cohesive Termini

To 11 µl of the reaction product from Section 1.E.(g) above, 10 µl of a solution containing 50 mM Tris, pH 7.5, 10 mM $MgSO_4$, 200 mM NaCl were added. T4 DNA ligase was heat inactivated by incubation at 70° C for 10 min. Forty units of EcoRI were added and the incubation was carried out at 37° C for 3 hr. The reaction was terminated by addition of EDTA to 50 mM. The sample was clarified by centrifugation and applied tc an AcA 34 column.

(i) AcA 34 Column Chromatography

Free linkers (those not ligated to ds cDNA) were removed from ds cDNA with attached linkers, to

prevent them from interfering with the insertion of the desired ds cDNAS into the cloning vectors. AcA 34 resin (a mixture of acrylamide and agarose beads, normally used for sizing) preswollen in 2 mM citrate buffer and 0.04% sodium azide in water, was added to the 1 ml mark of a 1 ml plastic syringe plugged with glass wool. The column was equilibrated with 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 400 mM NaCl. The ds cDNA mixtures with ligated linkers and free linkers (~45 µl) was brought to 400 mM NaCl. 1 µl of 0.5% bromophenol blue dye (BPB) was added, and the sample was applied to the column which was run in equilibration buffer at room temperature. Ten 200 µl fractions were collected. The BPB dye normally eluted from the column in the sixth tube or later. Tubes 1 and 2 were combined and used as the source of ds cDNA for cloning.

(j) Assembly of λgt10 clones

The ds cDNA was mixed with 1 µg of EcoRI-cut λgt10 DNA, precipitated with ethanol, and centrifuged. After washing the pellet once with 70% ethanol, the DNA pellet was air dried and resuspended in 4.5 µl of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl. To anneal and ligate the cDNA inserts to the left and right arms of the λgt10 DNA, the mixture was heated at 70° C for 3 min., then at 50° C for 15 min. The mixture was chilled on ice and 0.5 µl each of 10 mM ATP, 0.1 M DTT, and sufficient T4 DNA ligase to ensure at least 90% completion were added. The reaction was incubated at 14° C overnight, which allowed the insertion of the ds cDNA into the EcoRI site of the λgt10 DNA. The resulting DNA was packaged into phage particles *in vitro* using the method described by Scherer 1981.

(k) Removal of Phages Without Inserts

Insertion of a cDNA into the EcoRI site of λgt10 results in inactivation of the CI gene. λgt10 phages with inactivated C1 genes (i.e., with inserts) replicate normally in *E. coli* MA150 cells. By contrast, λgt10 phages without inserts are unable to replicate in the MA150 strain of *E. coli*. This provides a method of removing λgt10 clones which do not have inserts.

The phages in the library were first replicated in *E. coli* C600 (M^+R^-) cells which modified the λgt10 DNA to protect it from the *E. coli* MA150 restriction system. A relatively small number of *E. coli* C600 cells were infected and then plated with a 20 fold excess of MA150 (M^+R^+) cells. The primary infection thus occurred in the M^+R^- cells where all the phages will grow, but successive rounds of replication occurred in the MA150 cells which prevented the replication of phages without inserts. The amplified phage library was collected from the plates, and after removal of agar and other contaminants by centrifugation, the recombinant phages were ready to use in screening experiments.

F. Screening of cDNA Library; Selection of pMON9531

Approximately 600 phages (each plate) were spread on 10 cm x 10 cm square plates of solid NZY agar (Maniatis 1982) with 0.7% agarose. A translucent lawn of *E. coli* MA150 cells were growing on the plates. Areas where the phages infected and killed the *E. coli* cells were indicated by clear areas called "plaques," which were visible against the lawn of bacteria after an overnight incubation of the plates at 37° C. Six plates were prepared in this manner. The plaques were pressed against pre-cut nitrocellulose filters for about 30 min. This formed a symmetrical replica of the plaques. To affix the phage DNA, the filters were treated with 0.5 M NaOH and 2.5 M NaCl for 5 min. The filters were then treated sequentially with 1.0 M Tris-HCl, pH 7.5 and 0.5 M Tris-HCl, pH 7.5 containing 2.5 M NaCl to neutralize the NaOH. They were then soaked in chloroform to remove bacterial debris. They were then air-dried and baked under a vacuum at 80° C for 2 hours, and allowed to cool to room temperature. The filters were then hybridized with ^{32}P-labelled EPSP-1 probe (2 x 10^6 cpm/filter) as described in Section 1.D(e) above. After 48 hr of hybridization, the filters were washed in 6x SSC at room temperature twice for 20 min and then at 37° C for 5 min. These washes removed non-specifically bound probe molecules, while probe molecules with the exact corresponding sequence (which was unknown at the time) remained bound to the phage DNA on the filter. The filters were analyzed by autoradiography after the final wash. After the first screening step, seven positively hybridizing signals appeared as black spots on the autoradiograms. These plaques were removed from the plates and replated on to fresh plates at a density of 100-200 plagues/plate. These plates were screened using the procedure described above. Four positively hybridizing phages were selected. DNA was

isolated from each of these four clones and digested with EcoRI to determine the sizes of the cDNA inserts. The clone containing the largest cDNA insert, approximately 330 bp, was selected, and designated λE3. The cDNA insert from λE3 was inserted into plasmid PUC9 (Vieira 1981), and the resulting plasmid was designated pMON9531.

To provide confirmation that the pMON9531 clone contained the desired EPSP synthase sequence, the insert was removed from the pMON9531 clone by digestion with EcoRI. This DNA fragment was then sequenced by the chemical degradation method of Maxam (1977). The amino acid sequence deduced from the nucleotide sequence corresponded to the EPSP synthase partial amino acid sequence shown in Table 1.

G. Creation of AF7 Genomic DNA Clone

In order to obtain the entire EPSP synthase gene, chromosomal DNA from the MP4-G cells line was digested with BamHI and cloned into a phage vector to create a library, which was screened using the partial EPSP synthase sequence from pMON9531 as a probe.

(a) Preparation of MP4-G Chromosomal DNA Fragments

MP4-G cells were frozen and pulverized in a mortar with crushed glass in the presence of liquid nitrogen. The powdered cells were mixed with 8 ml/g of cold lysis buffer containing 8.0M urea, 0.35M NaCl, 0.05M Tris-HCl (pH 7.5), 0.02M EDTA, 2% sarkosyl and 5% phenol. The mixture was stirred with a glass rod to break up large clumps. An equal volume of a 3:1 mixture of phenol and chloroform containing 5% isoamyl alcohol was added. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%. The mixture was swirled on a rotating platform for 10-15 minutes at room temperature. The phases were separated by centrifugation at 6,000 x g for 15 minutes. The phenol/chloroform extraction was repeated. Sodium acetate was added to the aqueous phase to a final concentration of 0.15 M and the DNA was precipitated with ethanol. The DNA was collected by centrifugation, dissolved in 1x TE (10mM Tris-HCl, pH 8.0, 1 mM EDTA) and banded in a CsCl-ethidium bromide gradient. The DNA was collected by puncturing the side of the tube with a 16 gauge needle. The ethidium bromide was extracted with CsCl-saturated isopropanol, and the DNA was dialyzed extensively against 1x TE. Approximately 400 µg of DNA was isolated from 12 g of cells.

MP4-G chromosomal DNA (10 µg) was digested to completion with 30 units of BamHI in a buffer containing 10 mM Tris, pH 7.8, 1 mM DTT, 10mM $MgCl_2$, 50 mM NaCl for 2 hours at 37° C. The DNA was extracted with phenol followed by extraction with chloroform and precipitated with ethanol. The DNA fragments were suspended in 1x TE at a concentration of 0.5 µg/µl.

(b) Cloning of MP4-G Chromosomal DNA Fragments in λMG14

DNA from phage λMG14 (obtained from Dr. Maynard Olson of the Washington University School of Medicine, St. Louis, Missouri) was prepared by the method described in Maniatis 1982. 150 µg of DNA was digested to completion with BamHI in a buffer containing 10mM Tris-HCl, pH 7.8, 1 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl. The completion of the digest was checked by electrophoresis through 0.5% agarose gel. The phage DNA was then extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. The DNA was resuspended in 1x TE at a concentration of 150 µg/ml. $MgCl_2$ was added to 10 mM and incubated at 42° C for 1 hr to allow the cohesive ends of λDNA to reanneal. Annealing was checked by agarose gel electrophoresis.

After annealing, DNA was layered over a 38 ml (10-40%, w/v) sucrose gradient in a Beckman SW27 ultracentrifuge tube. The gradient solutions were prepared in a buffer containing 1 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA. 75 µg of DNA was loaded onto each gradient. The samples were centrifuges at 26,000 rpm for 24 hours at 15° C in a Beckman SW 27 rotor. Fractions (0.5 ml) were collected from the top of the centrifuge tube and analyzed for the presence of DNA by gel electrophoresis. The fractions containing the annealed left and right arms of λDNA were pooled together, dialyzed against TE and ethanol-precipitated. The precipitate was washed with 70% ethanol and dried. The DNA was dissolved in TE at a concentration of 500 µg/ml.

The purified arms of the vector DNA and the BamHI fragments of MP4-G DNA were mixed at a molar

ratio of 4:1 and 2:1 and ligated using T4 DNA ligase in a ligase buffer containing 66 mM Tris-HCl, pH 7.5,5 mM $MgCl_2$, 5 mM DTT and 1 mM ATP. Ligations were carried out overnight at 15° C. Ligation was checked by agarose gel eletrophoresis. Ligated phage DNA carrying inserts of MP4-G DNA were packaged into phage capsids *in vitro* using commercially available packaging extracts (Promega Biotech, Madison, WI). The packaged phage were plated in 10 cm x 10 cm square plates of NZY agar in 0.7% agarose at a density of approximately 6000 plaques per plate using *E. coli* C600 cells. After overnight incubation at 37° C, the plaques had formed, and the plates were removed from the incubator and chilled at 4° C for at least an hour. The agar plates were pressed against nitrocellulose filters for 30 minutes to transfer phages to the filters, and the phage DNA was affixed to the filters as described previously. Each filter was hybridized for 40 hours at 42° C with approximately 1.0 x 10^5 cpm/filter of the 330 bp cDNA insert isolated from the pMON9531 clone, which had been nick-translated, using the procedure described by Maniatis (1982). The specific activity of the probe was 2-3 x 10^8 cpm/µg of DNA. Hybridization was carried out in a solution containing 50% formamide, 5x SSC, 5x Denhardt's solution, 200 µg/ml tRNA and 0.1% SDS. Filters were washed in 1x SSC, 0.2% SDS at 50° C and autoradiographed. Several positive signals were observed and matched with plaques on the corresponding plate. The selected plaques were isolated from the plates, suspended in SM buffer, and plated with NZY agar. The replica plate screening process was repeated at lower densities until all the plaques on the plates showed positive signals. One isolate was selected for further analysis and was designated as the λF7 phage clone.

H. Preparation of pMON9543 and pMON9556

The DNA from λF7 was digested (separately) with BamHI, BglII, EcoRI, and HindIII. The DNA was hybridized with a nick-translated EPSP synthase sequence from pMON9531 in a Southern blot procedure. Results from that experiment indicated that the complementary sequence from λF7 was on a 4.8 kb BglII fragment. This fragment was inserted into plasmid pUC9 (Vieira 1982), replicated, nick-translated, and used to probe the petunia cDNA library, using hybridization conditions as described in Section 1.(G) and 10^6 cpm per filter. A cDNA clone with a sequence that bound to the λF7 sequence was identified, and designated as pMON9543.

DNA sequence analysis (Maxam 1977) indicated that pMON9543 did not contain the stop codon or the 3′ non-translated region of the EPSP synthase gene. Therefore, the EPSP synthase sequence was removed from pMON9543, nick-translated, and used as a probe to screen the cDNA library again. A clone which hybridized with the EPSP synthase sequence was identified and designated as pMON9556. DNA sequence analysis indicated that the insert in this clone contained the entire 3′ region of the EPSP synthase gene, including a polyadenylated tail. The 5′ EcoRI end of this insert matched the 3′ EcoRI end of the EPSP synthase insert in pMON9531. An entire EPSP synthase coding sequence was created by ligating the EPSP synthase inserts from pMON9531 and pMON9556.

I. Preparation of pMON546 Vector with CaMV35S/EPSP Synthase Gene

The EPSP synthase insert in pMON9531 was modified by site-directed mutagenesis (Zoller et al, 1983) using an M13 vector (Messing 1981 and 1982) to create a BglII site in the 5′ non-translated region of the EPSP synthase gene. The modified EPSP synthase sequence was isolated by EcoRI and BglII digestion, and inserted into vector, pMON530, a binary vector for *Agrobacterium*-based plant transformation to obtain pMON536. The 1.62 kb EcoRI-EcoRI fragment from pMON9556 was then inserted into pMON536 to obtain pMON546. Since pMON530 already contained a 35S promoter from a cauliflower mosaic virus (CaMV) next to the BglII site, this created a chimeric CaMV35S/EPSP synthase gene in pMON546.

pMON530, a derivative of pMON505 carrying the 35S-NOS cassette, was prepared in the following manner:

The CaMV35S promoter was isolated from the pOS-1 clone of CM4-184 as an AluI (n 7143)-EcoRI* (n 7517) fragment which was inserted first into pBR322 cleaved with BamHI, treated with Klenow fragment of DNA polymerase I and then cleaved with EcoRI. The promoter fragment was then excised from pBR322 with BamHI and EcoRI, treated with Klenow polymerase and inserted into the SmaI site of M13mp8 so that the EcoRI site of the mp8 multi-linker was at the 5′ end of the promoter fragment. The nucleotide numbers refer to the sequence of CM1841 (Gardner et al., 1981). Site directed mutagenesis was then used to introduce a G at nucleotide 7464 to create a BglII site. The CaMV35S promoter fragment was then excised from the M13 as a 330 bp EcoRI-BglII fragment which contains the CaMV35S promoter, transcription

initiation site and 30 nucleotides of the 5′ non-translated leader but does not contain any of the CaMV translational initiators nor the CaMV35S transcript polyadenylation signal that is located 180 nucleotides downstream from the start of transcription (Covey et al., 1981; Guilley et al., 1982). The CaMV35S promoter fragment was joined to a synthetic multi-linker and the NOS 3′ non-translated region and inserted into pMON200 (Fraley et al., 1985; Rogers et al., 1986) to give pMON316 (see Rogers et al., 1987).

Plasmid pMON316 contains unique cleavage sites for BglII, ClaI, KpnI, XhoI and EcoRI located between the 5′ leader and the NOS polyadenylation signals. Plasmid pMON316 retains all of the properties of pMON200. The complete sequence of the CaMV35S promoter, multi-linker and NOS 3′ segment is given in Rogers et al., 1987. This sequence begins with the XmnI site created by Klenow polymerase treatment to remove the EcoRI site located at the 5′ end of the CaMV35S promoter segment.

Plasmid pMON530 (Rogers et al., 1987) is a derivative of pMON505 prepared by transferring the 2.3 kb StuI-HindIII fragment of pMON316 into pMON526. Plasmid pMON526 is a simple derivative of pMON505 in which the SmaI site is removed by digestion with XmaI, treatment with Klenow polymerase and ligation. Plasmid pMON530 retains all the properties of pMON505 and the CaMV35S-NOS expression cassette and now contains a unique cleavage site for SmaI between the promoter and polyadenylation signal.

Binary vector pMON505 is a derivative of pMON200 in which the Ti plasmid homology region, LIH, has been replaced with a 3.8 kb HindIII to SmaI segment of the mini RK2 plasmid, pTJS75 (Schmidhauser & Helinski, 1985). This segment contains the RK2 origin of replication, oriV, and the origin of transfer, oriT, for conjugation into *Agrobacterium* using the tri-parental mating procedure (Horsch & Klee, 1986).

Referring to Figure 6, plasmid pMON505 retains all the important features of pMON200 including the synthetic multi-linker for insertion of desired DNA fragments, the chimeric NOS-NPTII′-NOS kanamycin resistance determinant for selection in transgenic plants and a streptomycin/spectinomycin gene for selection in *E. coli* and *A. tumefaciens*, an intact nopaline synthase gene for facile scoring of transformants and inheritance in progeny and a pBR322 origin of replication for ease in making large amounts of the vector in *E. coli*. Plasmid pMON505 contains a single T-DNA border derived from the right end of the pTiT37 nopaline-type T-DNA. Southern analyses have shown that plasmid pMON505 and any DNA that it carries are integrated into the plant genome, that is, the entire plasmid is the T-DNA that is inserted into the plant genome. One end of the integrated DNA is located between the right border sequence and the nopaline synthase gene and the other end is between the border sequence and the pBR322 sequences.

Plasmid pMON546 contained (1) the CaMV35S/EPSP synthase gene; (2) a selectable marker gene for kanamycin resistance (Kan^R); (3) a nopaline synthase (NOS) gene as a scorable marker; and (4) a right T-DNA border, which effectively caused the entire plasmid to be treated as a "transfer DNA" (T-DNA) region by *A. tumefaciens* cells.

This plasmid was inserted into *A. tumefaciens* cells which contained a helper plasmid, pGV3111-SE. The helper plasmid encodes certain enzymes which are necessary to cause DNA from pMON546 to be inserted into plant cell chromosomes. It also contains a kanamycin resistance gene which functions in bacteria.

A culture of *A. tumefaciens* containing pMON546 and pGV3111-SE was deposited with the American Type Culture Collection (ATCC) and was assigned ATCC accession number 53213. If desired, either one of these plasmids may be isolated from this culture of cells using standard methodology. For example, these cells may be cultured with *E. coli* cells which contain a mobilization plasmid, such as pRK2013 (Ditta 1980). Cells which become Spc/Str^R, Kan^S will contain pMON546, while cells which become Kan^R, $Spc\,Str^S$ will contain pGV3111-SE.

GLYPHOSATE-TOLERANT PETUNIA PLANTS

Leaf disks with diameters of 6 mm (1/4 inch) were taken from surface-sterilized petunia leaves. They were cultivated on MS104 agar medium for 2 days to promote partial cell wall formation at the wound surfaces. They were then submerged in a culture of *A. tumefaciens* cells containing both pMON546 and GV3111-SE which had been grown overnight in Luria broth at 28° C, and shaken gently. The cells were removed from the bacterial suspension, blotted dry, and incubated upside down on filter paper placed over "nurse" cultures of tobacco cells, as described by Horsch (1980). After 2 or 3 days, the disks were transferred to petri dishes containing MS media with 500 µg/ml carbenicillin and 0, 0.1, 0.25, or 0.5 mM glyphosate (sodium salt), with no nurse cultures.

Control tissue was created using *A. tumefaciens* cells containing the helper plasmid pGV3111-SE and a different plant transformation vector, pMON505, which contained a T-DNA region with a NOS/NPTII/NOS

kanamycin resistance gene and a NOS selectable marker gene identical to pMON546, but without the CaMV35S/EPSP synthase gene.

Within 10 days after transfer to the media containing glyphosate, actively growing callus tissue appeared on the periphery of all disks on the control plate containing no glyphosate. On media containing 0.1 mM glyphosate, there was little detectable difference between the control disks and the transformed tissue. At 0.25 mM glyphosate, there was very little growth of callus from control disks, while substantial growth of transformed tissue occurred. At 0.5 mM glyphosate, there was no callus growth from the control disks, while a significant number of calli grew from the transformed disks. This confirms that the CaMV35S/EPSP synthase gene conferred glyphosate resistance upon the transformed cells.

Transformed petunia plants were produced by regeneration from the above-described transformed leaf disks by the procedure described by Horsch, et al. (1985). The transformed plants obtained contained the pMON546 vector, described hereinabove, which contains the CaMV 35S promoter fused to the wild-type petunia EPSP synthase gene.

Four individual representative transgenic seedlings were selected, grown and tested in the testing procedure described below, along with four individual non-transformed (wild-type) petunia seedlings.

The plants were grown in a growth medium in a growth chamber at 26° C with 12 hours of light per day. The plants were fertilized weekly with a soluble fertilizer and watered as needed. The plants were sprayed at a uniform and reproducible delivery rate of herbicide by use of an automated track sprayer. The glyphosate solution used was measured as pounds of glyphosate acid equivalents per acre, mixed as the glyphosate isopropylamine salt, with an ionic surfactant.

Four individual wild-type (non-transformed) petunia plants were selected for use as control plants. Four individual transformed plants containing the pMON546 vector were selected by kanamycin resistance as described by Horsch, et al. (1985).

The control plants and the transformed plants were sprayed with the isopropylamine salt of glyphosate at the application level listed in Table 2 below; the experimental results obtained are also summarized in Table 2.

Table 2

Plant Response to Glyphosate Spraying		
Plant Type	Glyphosate Dose *	Visual Appearance
Control[1]	0.897 kg/hectare (0.8 #/acre)	completely dead, plants showed very rapid chlorosis and bleaching, wilted and died
Chimeric EPSP	0.897 kg/hectare (0.8 #/acre)	growing well, showed slight chlorosis in new leaves which are growing with normal morphology, plants appear healthy and started to flower

* Acid Equivalent

[1] wild-type plant or transformed with control vector (pMON505)

As indicated in Table 2, the control plants were killed when sprayed with 0.897 kg/hectare (0.8 pounds/acre) of glyphosate. In contrast, the petunia plants which were transformed were healthy and viable after spraying with 0.897 kg/hectare (0.8 pounds/acre). The transformed plants are more resistant to glyphosate exposure than the non-transformed control plants.

Glyphosate-Tolerant Petunia EPSP Synthase

A plant transformation vector carrying a glycine (101) to alanine petunia EPSP synthase variant was prepared in the following manner.

Plasmid pMON530 DNA was digested with BglII and ClaI, to which was added the 330 bp BglII-EcoRI EPSP synthase 5' fragment from pMON536 and purified 1.4 kb EcoRI-ClaI EPSP synthase 3' fragment from pMON9566 and then treated with T4 DNA ligase. Following transformation a plasmid was isolated that carried the intact glycine (101) to alanine variant EPSP synthase coding sequence of petunia (with the

coding sequence for the chloroplast transmit peptide) adjacent to the CaMV35S promoter. This plasmid was designated pMON567. Plasmid pMON567 was inserted into A. tumefaciens cells that contained helper plasmid pGV3111-SE.

A culture of A. tumefaciens cells containing pMON567'pGV3III-SE was contacted with leaf disks taken from tobacco plants (Nicotiana tobacam CV H425) as described by Horsch (1985). The Agrobacterium cells inserted the variant EPSP synthase gene into the chromosomes of the plant cells. Plant cells resistant to kanamycin were selected and regenerated into differentiated plants by the procedure of Horsch (1985).

Progeny of these plants were propagated and grown to a rosette diameter of about 10 cm corresponding to a plant age of about four weeks. The plants were sprayed with glyphosate at levels corresponding to 0.448 kg/hectare, 2.242 kg/hectare, and 4.035 kg/hectare (0.4, 2.0 and 3.6 pounds acid equiv./ acre). The effect of glyphosate on the transformed plants were scored at 7, 14 and 28 days. The effect was translated to a numerical scale of 0-10 in which 0 represents total kill and 10 is the normal, unsprayed plant. The data below demonstrates that tobacco plants transformed with the glyphosate-tolerant EPSP synthase gene of petunia exhibit substantial tolerance even to these high levels of glyphosate. The values represent the best transformant for both wild-type EPSP synthase and glyphosate-tolerant EPSP synthase genes.

Table 3

Relative Effect of Glyphosate [1]						
Day	kg/hectare (pounds/Acre)					
	0.448 (0.4)		2.242 (2.0)		4.035 (3.6)	
	GT[2]	WT[3]	GT	WT	GT	WT
7	8.0	6.0	8.0	5.0	5.0	5.0
14	8.0	7.0	8.3	1.8	7.4	1.7
28	9.0	9.0	7.0	0.8	7.0	0.8

[1] 0 represents total kill and 10 represents no effect.
[2] Glyphosate-tolerant petunia EPSP synthase.
[3] Wild-type EPSP synthase.

II. EPSP SYNTHASE cDNA CLONE OF TOMATO

Complementary DNA (cDNA) libraries were prepared from poly-A plus RNA isolated from mature tomato pistils or anthers by a modification of the methods of Huynh et al. (1985) and Gubler et al. (1983) as follows:

First Strand Synthesis

Quantities given below are those used to prepare the mature pistil cDNA library, the anther cDNA library was prepared in a similar manner.

10µl of 400µg/ml Actinomycin D (Sigma Chemical) in 50% ethanol was dried down in each reaction tube in a Savant speed vacuum. The following reagents were added to this tube (the reagents were added in the order given):

Vol.	Substance	Final Conc/Amount
62 µl	Autoclaved water	to final 100 µl
10 µl	10 X first strand buffer	see below
10 µl	5 mM dNTP	500 µM each A,C,G,T[1]
10 µl	100 µg/ml	oligo p(dT)1 µg[2]
2 µl	RNAsin (30 U/µl)	60 U[3]
2 µl	RNA	~1.5 µg
3 µl	Reverse Transcriptase	40 units[4]
1 µl	^{32}P-dCTP	20 µCi[5]

[1] Sigma Chemical, St. Louis, MO.
[2] Collaborative Research, Lexington, MA.
[3] Promega Biotech, Madison, WI.
[4] Life Sciences, St. Petersburg, FL.
[5] Amersham, Arlington Heights, IL.

The reaction mixture was incubated at 42° C for 60 min. The reaction mixture was frozen on dry ice and stored at -20° C.

10 X First Strand buffer
500 mM Tris-HCl pH 8.3
300 mM KCl
100 mM $MgCl_2$
4 mM Dithiothreitol, DTT

The quantity of cDNA synthesized was determined to be ~1.31 µg by precipitation of a portion of the reaction with trichloroacetic acid and scintillation counting.

Purification of First Strand

Biogel P60 (100-200 mesh, Bio Rad, Richmond, CA), pre-swollen in 10 mM Tris-HCl/1 mM EDTA, pH 8.0, (TE) was used to pour a column in a siliconed pasteur pipet plugged with silicon-treated glass wool (bed volume = 1 ml). The column was washed with several volumes of 1 mM Tris pH 7.6/ 0.01 mM EDTA. The column was calibrated by running 90 µl of this same solution plus 10 µl of column marker buffer (see below) over the column. The void volume was determined by the fraction containing the blue dye. More buffer was added to the column to elute the red dye.

The first strand reaction was extracted twice with an equal volume of phenol. 0.5 µl 2% bromophenol blue was added to the cDNA and it was loaded on the column, and the void volume was collected.

Column Marker Buffer:
5% Blue Dextrans (2 M dalton, Sigma)
0.05% Phenol Red (or Bromophenol blue at 0.1%)
dissolved in 20 mM Tris pH 7-8/ 1 mM EDTA

Second Strand Synthesis and Methylation

The first strand was dried to approximately 10 µl in a Savant speed vacuum.

Vol.	Substance	First Conc. Amount
3.8 µl	cDNA	~500 ng of first strand
10 µl	10X Sec. Strand Buffer	1 X
0.8 µl	5 mM dNTP	40 µM each 81.5 µl
	Water	to 100 µl final volume
2 µl	DNA Pol I (NEB)	20 U
0.4 µl	*E. coli* DNA ligase (NEB)	2 U
0.5 µl	RNAase H (BRL)	1 U
3 µl	32P dCTP	30 uCi
1 µl	BSA (1:10 dil of BRL)	50 µg/ml
NEB = New England Biolabs, Beverly, MA BRL = Bethesda Research Labs, Gaithersberg, MD		

The reaction was incubated at 14° C for 60 min. then at room temperature for 60 min.

The following was added:

0.5 µl 5mM dNTP

1 µl T4 DNA polymerase (NEB)

The reaction was incubated for 30 min. at room temperature.

The following were added:

1.2 µl 1 mM S-adenosyl L-methionine (Sigma) 12 µM

1.0 µl EcoRI Methylase (NEB) 20 U

2.4 µl 0.5 M EDTA 12 mM

5 µl was removed from the reaction and added to 260 ng wild type lambda DNA (NEB) as control for methylation.

The reactions were incubated at 37° C for 45 min.

Both the main and test reactions were heated to 68° C for 10 min. to inactivate enzymes.

Measurements of trichloroacetic acid insoluble counts indicated that ~500 ng of ds cDNA (double stranded cDNA) was produced in the reaction.

10X Second Strand Buffer:		
200 mM	Tris-HCl pH 7.4-7.5	1 M stock
50 mM	$MgCl_2$	1 M stock
1.0 M	KCl	4 M stock
100 mM	Ammonium sulfate	1 M stock
1.5 mM	Beta-NAD	150 mM stock

Assay for Completeness of Methylation

The following was added to the heat treated test methylation:

2 pl 100 mM Tris-HCl pH 7.6/100 mM $MgCl_2$/1.0 M NaCl

12 µl water

1 µl EcoRI (20 units BRL)

0.5 µl pUC19 (0.5 µg, NEB)

The reaction was incubated for 1 hr. at 37° C.

The products were run on an agarose minigel with undigested pUC19, and lambda digested with EcoRI and HindIII as size markers. The pUC19 in the reaction digested to completion, indicating that the EcoRI was working efficiently and the lambda DNA was undigested, showing that it had been protected by the methylation reaction. This shows that the methylase was effective in blocking the EcoRI sites in the cDNA from digestion.

ds cDNA Clean Up

The second strand reaction mixture was extracted twice with an equal volume of phenol, run over a P-60 column as described above and the void volume was collected and lyophilized in a Savant speed vacuum. The cDNA was dissolved in 3 µl of 1 mM Tris-HCl pH 7.5/0.01 mM EDTA.

Ligation of Linkers to cDNA

The following was mixed in a microfuge tube:
3 µl ds cDNA (500 ng)
2.5 pl Phosphorylated EcoRI linkers (NEB, 250 ng)
1 µl 10 x Ligation buffer
1 µl 10 mM ATP
1.5 µl water (for final vol of 10 µl)
1 µl T4 DNA Ligase (~400 units NEB)
The reaction was incubated at 14° C for 12 hr.
10 x Ligation Buffer
300 mM Tris-HCl pH 7.6
100 mM $MgCl_2$
50 mM DTT

Removal of Linkers

The following reagents were added:
2 µl 100 mM Tris-HCl pH 7.6/100 mM $MgCl_2$/1.0M NaCl
6 µl water
The reaction was heated to 68° C for 10 min. to inactivate ligase.
The following reagent was added:
2 µl EcoRI (40 units, NEB)
The reaction was incubated at 37° C for 2.5 hr.
The reaction was heated to 68° C for 10 min. to inactivate EcoRI.

Size Cut cDNA and Separate From Linkers

5 µl of loading buffer was added to the digested cDNA/EcoRI linker reaction. The sample was electrophoresed on a 0.8% Sea Plaque agarose (FMC Corp., Rockland, MD)/TEA (40 mM Tris-Acetate pH 8.2/1.6 mM EDTA) mini-gel containing 0.3 µg/ml ethidium bromide. The gel was run at 4 V/cm until the bromophenol blue dye had migrated 4 cm. Lambda DNA digested with HindIII and EcoRI was used as a size marker. The markers were visualized by UV fluorescence, and a fragment of gel containing cDNA ranging in size from ~600 bp to greater than 10 kb was removed.
Loading Buffer:
250 mM EDTA pH 7
0.2% Bromophenol blue 50% Glycerol

Purification, Ligation and Packaging

The volume of the gel slice was determined to be ~500 µl by weighing and assuming a density of 1.0 g/ml, 140 µl of 20 mM Tris-HCl (pH 7.5)/200 mM NaCl/1.0 mM EDTA and 20 µl of 5 M NaCl were added to the gel fragment. The mixture was heated to 68° C for 15 min. and extracted twice with 500 µl of phenol. The DNA was purified from contaminants by chromatography on an EluTip D column (Schleicher & Schuell, Keen, NH) according to the manufacturers instructions. The final volume was 450 µl. The amount of radioactivity in the sample was determined by scintillation counting of an aliquot, and it was determined that 70 ng of cDNA was contained in the eluted volume.

2 µl (2 ug) lambda gt 10 arms (Vector Cloning Systems, San Diego, CA) were added to the cDNA

followed by the addition of 2 volumes of cold ethanol. The sample was chilled to -80° C for 15 min. and the precipitate was pelleted in a microfuge for 15 min. The tube was drained and rinsed with 200 µl of -20° C 70% ethanol with caution so as not to disturb the pellet. The pellet was air dried for 30 min.

The following was added:

7.2 µl Water
1 µl 10 X Ligation buffer
1 µl ATP
0.8 µl T4 DNA ligase

The reaction was incubated for 20 hrs at 14° C.

10 x Ligation Buffer
200 mM Tris-HCl pH 7.6
100 mM $MgCl_2$
50 mM Dithiothreitol (DTT)

One fourth (2.5 µl) of the ligation reaction was packaged *in vitro* into phage using Gigapack packaging extracts (Stratagene Cloning Systems, San Diego, CA) according to the manufacturers instructions. Subsequent plating of the phage showed that this reaction contained 10^6 recombinant plaque forming units (PFU). Packaging of the entire ligation mix would therefore produce 4 x 10^6 PFU. The remainder of the ligation mix was stored at -20° C for future use.

Plaque lifts from the two libraries were screened with a ^{32}P-labeled fragment from pMON6145 containing the complete coding sequence of petunia EPSP synthase. pMON6145 is a derivative of plasmid pGEM2 (Promega Biotech, Madison, WI) described in the above-referenced and incorporated application S.N. 879,814, which carries a full-length cDNA clone of petunia EPSP synthase. Two hybridizing plaques were isolated from each library. The large EcoRI fragments of the two pistil clones (P1 and P2) were subcloned into pUC19 (New England Biolabs), and the small EcoRI fragments were cloned into pUC119 forming plasmids 9591, 9589, 9595 and 9596, respectively.

pUC119 is constructed by isolating the 476 bp Hgi AI/Dra I fragment of bacteriophage M13 and making the ends of the fragment blunt with T4 DNA polymerase (New England Biolabs). This fragment is then inserted into pUC19 (Yanisch-Perron et al., 1985) that has been digested with Nde I and filled in with Klenow DNA polymerase (New England Biolabs). The resulting plasmid (pUC119) can be used to produce single stranded DNA if cells harboring the plasmid are infected with a defective phage such as R408 (Stratagene Cloning Systems).

In order to introduce an NcoI site and an ATG translational initiation codon at the site predicted to be the start of the mature enzyme for in vitro expression in *E. coli*, the 1.6 kb EcoRI/HindIII fragment of pMON9591 was cloned into EcoRI/HindIII digested M13mp18 (New England Biolabs) producing a phage designated M9568. This clone was mutagenized with the oligonucleotide:

5′-AGCACAATCTCATGGGGTTCCATGGTCTGCAGTAGCC-3′

as previously described. Sequencing confirmed the success of the mutagenesis and the resulting phage was designated M9575. The 1.6 kb EcoRI/HindIII fragment of this phage was inserted into EcoRI/HindIII digested pMON6140. This plasmid was designated pMON9717. Plasmid pMON6140 is a derivative of pGEM1 (Promega Biotech, Madison, WI) which carries the same full-length cDNA clone of petunia EPSP synthase as described above for pMON6145.

In vitro transcription and translation of pMON9717 failed to produce an active enzyme. Subsequent sequencing of the cDNA from which this clone was prepared (pMON9591) revealed a single nucleotide deletion in the coding sequence which would result in a frame shift in the coding sequence. The region containing this deletion was replaced by the corresponding region from pMON9589 by exchanging the 900 bp BamHI/HindIII fragment of pMON9717 with the corresponding fragment of pMON9589. This plasmid was designated pMON9718. *In vitro* analysis of pMON9718 showed it coded for active tomato EPSP synthase.

A vector for high level expression in *E. coli* was constructed to further characterize the tomato EPSP synthase. The NcoI/HindIII fragment of pMON9718 containing the coding sequence for tomato EPSP synthase was inserted into NcoI/HindIII digested pMON5521. This placed the tomato EPSP synthase coding sequence under the control of the *E. coli* RecA promoter (Horii et al., 1980: Sancar et al., 1980). This plasmid was designated pMON9719. Plasmid pMON9719 was able to complement the EPSP synthase deficiency of an *E. coli* aroA variant (SR481) demonstrating the synthesis of active EPSP synthase.

To introduce the alanine for glycine substitution at position 101 of tomato EPSP synthase, the wild-type EPSP synthase coding sequence in phage M9568 was mutagenized with the oligonucleotide.

5′ -GCCGCATTGCTGTAGCTGCATTTCCAAGG-3′

by method of Zoller and Smith (1983) as described previously. The phage is then mutagenized with the obligonucleotide.

5′ -CTCATCCTAGGAACGTCATCAAGAACATA-3′
to introduce the aspartate for glycine change at position 144 of the mature enzyme. A plant transformation vector capable of producing the glyphosate resistant form of tomato EPSP synthase in transgenic plants is constructed as follows:

A BglII site is engineered upstream of the ATG translation initiation codon of tomato pre-EPSP synthase by performing site directed mutagenesis on pMON9596. The mutagenesis is performed by the method of Kunkel (1985) using the oligodeoxynucleotide.
5′ -GCCATTTCTTGTGAAAAAAGATCTTTCAGTTTTTC-3′

The 700 bp EcoRI/BamHI fragment of the phage which had been engineered to include the glycine (101) to alanine and glycine (144) to aspartic acid changes is then transferred into EcoRI/BamHI digested pMON9718 replacing the corresponding wild-type fragment.

The 70 bp BG1II/Eco RI fragment of the altered pMON9596 is then combined with the 1.6 kb EcoRI/HindIII fragment of the M9718 derivative into Bg1II/HindIII digested pMON550. pMON550 is a derivative of pUC19 (Yanisch-Perron et al. 1985) produced by inserting the synthetic DNA fragment.

```
5' -AGCTTTCTAGAAGATCTCCATGGAGGCCTGGTAC-3'
3' -AAGATCTTCTAGAGGTACCTCCGGAC-5'
```

into pUC19 digested with HindIII and Kpn I. This reconstitutes a complete tomato EPSP synthase precursor gene which includes the alanine for glycine substituion.

For insertion into a plant transformation vector a convenient site is engineered at the 3′-end of the coding sequence by digestion with HindIII, making the ends blunt and inserting a ClaI linker (New England Biolabs). The 1.7 kb BglII/ClaI fragment of this plasmid is then inserted into Bg1II/ClaI digested plant transformation vector such as pMON316. The resulting plasmid has the tomato EPSP synthase precursor coding sequence with alanine for glycine substitution at position 101, and the glycine to aspartate substitution at position 144 of the mature EPSP synthase sequence under control of the CaMV35S promoter. Transformation of plants, such as tomato, with this vector leads to the production of a high level of the glyphosate-tolerant enzyme, resulting in glyphosate tolerant plants.

III. EPSP SYNTHASE GENOMIC CLONE OF *ARABIDOPSIS*

An *Arabidopsis thaliana* genomic bank was prepared by cloning size fractionated (15-20 kb) MboI partially digested DNA into BamHI and EcoRI digested lambda EMBL3 (Strategene Cloning Systems, San Diego, CA). Approximately 10,000 plaques of phage from this library were screened with ^{32}P labeled petunia EPSP synthase probe (pMON9566 described hereinbefore). A strongly hybridizing plaque, designated E1, was purified. Southern blots of the phage DNA with the EPSP synthase probe identified two fragments which hybridized very strongly. The first fragment was a 1.0 kb HindIII fragment and the other was a 700 bp BamHI fragment. These fragments were subcloned into plasmid pUC119 and designated pMON574 and pMON578.

The DNA sequences for the two inserts were then determined by the method of Sanger (1977). The sequence data indicated that the phage did contain the EPSP synthase gene of *Arabidopsis* by its strong homology to the petunia EPSP synthase sequence. The 700 bp BamHI fragment was used as a hybridization probe against the phage and *Arabidopsis* genomic DNA to identify restriction fragments suitable for the cloning of the entire EPSP synthase gene. Two hybridizing BglII fragments of 6.0 kb and 3.2 kb were identified in the E1 phage clone. These fragments were separately subcloned into pMON550 to provide DNA for further experiments and designated pMON582 and pMON583, respectively. Two additional subclones were made from clones pMON582 and pMON583. Plasmid pMON584 is the 1.8 kb EcoRI to BamHI fragment containing the 5′-end of the *Arabidopsis* EPSP synthase gene in pUC118 which is prepared from pUC18 in a manner analogous to the preparation of pUC119 from pUC19 described hereinbefore. Plasmid pMON589 is the 2.8 kb BamHI to BlgII fragment containing the 3′-end of the *Arabidopsis* EPSP synthase gene in pUC119. Sequence determination from the BamHI site of pMON584, and from the BamHI site of pMON589 completed the sequence of the coding regions of the gene.

The coding sequence was altered so that the expressed *Arabidopsis* EPSP synthase would include the alanine for glycine substitution at position 101 of the mature enzyme. Plasmid pMON578 was mutagenized

with the oligonucleotide:
5′-CTTTACCTCGGTAATGCAGCTACAGCAATGCG-3′
by the method of Kunkel (1985). A portion of the resulting plasmid, pMON594, was sequenced to verify the mutation. pMON594 was then mutagenized with the oligonucleotide.
5′-TTGGTCTTAAGCAGCTTGACGCTGATGTTG-3′
by the method of Kunkel (1985) to introduce the aspartate for glycine mutation at position 144 of the mature enzyme. The resulting plasmid was partially sequenced to verify the success of the mutagenesis. This construct containing the internal 730 bp Bam HI fragment of the *Arabidopsis* EPSP synthase gene with the glycine (101) to alanine and glycine (144) to aspartic acid mutations was designated pMON9930.

A ClaI site is required just upstream of the translational initiation site for insertion of the *Arabidopsis* EPSP synthase gene into plant transformation/ expression vectors. A 370 bp SnaBI/BamHI fragment of pMON584 including the translational initiation site and 65 bp of 5′-untranslated region was cloned into EcoRV/BamHI digested Bluescript KS (Stratagene Cloning Systems, San Diego, California) forming pMON9734.

The entire *Arabidopsis* gene was reconstructed for plant transformation experiments as follows: the 3.0 kb BamHI to BglII fragment containing the 3′ half of the gene was excised from pMON583 and inserted into the unique BamHI site of pMON9734. This plasmid pMON588, has a unique BamHI site in the middle of the gene. The 800 bp BamHI fragment from pMON9930 was then inserted into the unique BamHI site of pMON588. This resulting plasmid, pMON982, contains the entire EPSP synthase gene with the alanine for glycine substitution at position 101 and the aspartic acid for glycine substitution at position 144 of the mature protein. pMON982 was digested with Cla I and treated with Klenow polymerase in the presence of dATP, dCTP, TTP, and dGTP to produce a blunt end. The plasmid was then digested with Eco RI and the 3.5 kb fragment containing the entire variant *Arabidopsis* EPSP synthase coding region with the alanine for glycine substitution at position 101 and the asparate for glycine substitution at position 144 of the mature protein is inserted into pMON979 under the control of the duplicated 35S promoter of CaMV (Kay et al., 1987). The resulting construct, pMON987 (Figure 5) is then introduced into *Agrobacterium tumefaciens* ACO as described hereinbefore.

The *Agrobacterium* harboring the pMON987 plasmid is used to transform explants of *Brassica napus* as described by the method of Fry et al. (1987). Gentamicin resistant plants are then obtained. The plants will have the pMON987 DNA integrated into their genomes and will be tolerant to glyphosate.

IV. EPSP SYNTHASE OF *GLYCINE MAX*

The cDNA for EPSP synthase of *Glycine max* (soybean) was isolated from a library constructed from RNA isolated from *G. max* root tips. The library was constructed using the commercially available Amersham cDNA synthesis kit (Amersham Corp., Arlington Hts., IL), and lambda gt10 from Vector Cloning Systems (San Diego, CA). The library was screened with an insert from pMON578 which contains part of the *Arabidopsis* EPSP synthase gene and hybridizing plaques were isolated and their inserts subcloned into Bluescript plasmids (Vector Cloning Systems, San Diego, CA), and single stranded phage. The sequence of a portion of one of the cDNA clones (pMON9752, containing a 1600 bp cDNA) was determined. Referring to Figure 2, the protein deduced from the nucleotide sequence has strong homology to the petunia sequence in the region corresponding to the mature protein. Notably, amino acids 94-107 of the petunia enzyme are identical to amino acids 97-110 of the mature *G. max* enzyme (a three amino acid insertion in the *G. max* relative to the petunia near the amino terminus of the mature protein is responsible for the difference in numbering).

The *Glycine max* enzyme was altered to change the glycine at position 104 (which corresponds to Gly 101 in petunia) to an alanine by site directed mutagenesis using the oligonucleotide:
5′-AAAGGACGCATTGCACTGGCAGCATTTCCAA-3′
according to the method of Kunkel (1985) resulting in plasmid pMON9923.

The gly 147 to asp mutation (corresponding to the petunia gly 144 to asp) was introduced into pMON9923 by site directed mutagenesis using the synthetic oligonucleotide primer.
5′ GCAATCAACATCTGCGTCAAGTTAA 3′
by the method of Kunkel (1985). The gly 104 to ala and gly 147 to asp mutations were confirmed by DNA sequence analysis. The resulting plasmid containing both mutations was designated pMON9952.

An *E. coli* expression vector for wild type soybean EPSPS was constructed by engineering an NcoI site at the predicted start of the mature soybean coding sequence by the method of Kunkel (1985). This

sequence was then inserted into pMON9563, replacing the coding sequence for mature petunia EPSPS resulting in pMON9904. To construct a vector for bacterial expression of the variant form of soybean EPSPS a KpnI/ClaI fragment of pMON9952 was used to replace the corresponding wild type fragment in pMON9904 resulting in pMON9953. pMON9953 was shown to complement the aroA defect of *E. coli* SR481. EPSP synthase extracted from these bacteria was shown to be glyphosate tolerant.

The original cDNA clone for soybean EPSPS did not contain the complete sequence for the transit peptide. To construct a vector for expression of the variant soybean EPSPS in higher plants a complete coding sequence was needed. A soybean genomic library was purchased from Clontech Inc. (Palo Alto, CA). The library was screened with a 32p-labelled probe made from the soybean cDNA. A hybridizing clone was isolated and a 5.5 kb SalI fragment of this phage was subcloned (pMON1608) and was shown to contain the entire transit peptide sequence. A BglII site was engineered just upstream of the start codon by oligonucleotide directed mutagenesis by the method of Kunkel (1985) resulting in pMON1618. A BglII fragment of pMON1618 was cloned into BglII digested pMON977, a plant transformation vector with the enhanced version of the 35S promoter (Kay et al., 1987) and the 3′ -end of the E9 rbcS gene of pea (Broglie et al., 1984) in the correct orientation for the 35S promoter to drive expression of EPSPS (pMON1619). To complete the construction of the plant expression vector the EcoRI fragment of pMON9952 containing the variant EPSPS mature coding sequence is used to replace the EcoRI fragment of pMON1619. The resulting plasmid is then introduced into plants which will exhibit enhanced tolerance to glyphosate herbicide.

V. EPSP SYNTHASE GENE FROM *BRASSICA NAPUS*

DNA was isolated from *Brassica napus* (c.v. Westar) by standard methods. The DNA was partially digested with the restriction endonuclease MboI (New England Biolabs). Fragments ranging in size from 12 to 24 kb were separated from smaller and larger fragments by electrophoresis on an agarose gel, and were isolated from the gel on DEAE membrane (Amersham). The fragments were ligated into the commercially available lambda cloning vector Lambda-Dash (Stratagene Inc.). Recombinant phage were plated and nitrocellulose replicas were made from the plates by standard methods. The filters were probed with an 850 bp HindIII fragment of the cDNA for *Arabidopsis* EPSP synthase which was isolated from a cDNA library by standard methods using probes made from the *Arabidopsis* EPSP synthase genomic clones described hereinbefore. The hybridizing clones were picked and rescreens were performed with the same probe and with a probe made from pMON9717, the tomato EPSP synthase cDNA described hereinbefore. Those clones hybridizing strongly to both probes were isolated and propagated for DNA purification. A 3.8 kb BglII fragment was cloned into pUC119 to form pMON663. Subclones of this plasmid were made and sequenced, positively identifying the clone as an EPSP synthase gene. The clone includes 270 bp of 5′ untranslated and flanking sequence, all eight exons and seven introns of the gene and ~800 bp of 3′-untranslated and flanking sequence.

To facilitate construction of plant transformation vectors a BglII site was introduced just upstream of the ATG translation initiation codon of the *B. napus* EPSP synthase gene by mutagenesis of pMON663 (Kunkel, 1985) with the primer:

5′-GCTAGATTGCGCCATAGATCTGAATTTGAAAGC-3′

The glycine to alanine change at position 101 of the mature *B napus* EPSP synthase was then introduced by similar mutagenesis of the resulting plasmid with the oligonucleotide:

5′-GGACGCATGGCTGTAGCTGCATTCCCAAG-3′

A third mutagenesis is then carried out to change the codon for amino acid 144 of the mature protein from glycine to aspartate using the oligonucleotide:

5′-ACTCAACATCAGCATCAAGCTGCTTAAG-3′

The resulting plasmid is then digested with BglII and EcoRI and the variant *B. napus* EPSP synthase gene is isolated and inserted into a plant transformation vector similar to pMON987 described hereinbefore. This plasmid is then introduced into an appropriate *Agrobacterium* strain such as ACO, described hereinbefore, and used to transform plants such as *Brassica napus*. Plants which have integrated this plasmid, or another similar plasmid constructed with this gene into their genomes will be tolerant to glyphosate.

VI. EPSP SYNTHASE GENE FROM MAIZE

Construction of a Glyphosate Tolerant Maize Gene

Maize seeds were imbibed for 12 hr in water, the embryos, including the scutella, were dissected from the seeds and RNA was purified from this material by the method of Rochester et al. (1986). polyA-mRNA was isolated from the RNA by chromatography on oligo dT cellulose, and was used to construct a cDNA library as described hereinbefore. The library was screened with a ^{32}-P labelled RNA probe synthesized *in vitro* from pMON9717 (tomato EPSP synthase cDNA) which had been linearized with HindIII. The probe was synthesized with T7 RNA polymerase (Promega, Madison, WI) according to the manufacturers instructions. Hybridizing plaques were isolated, replated and nitrocellulose lifts from the plates were screened with the same probe. Plaques representing single clones which hybridized strongly to the tomato probe were isolated, propagated and used to prepare DNA. A clone designated lambda-z1d was found to contain a 1.8 kb EcoRI insert. The insert of this phage was subcloned into the EcoRI site of Bluescript KS+ (Strategene, San Diego, CA) to form pMON9935. The complete sequence of this cDNA clone was determined and used to deduce the amino acid sequence shown in Figure 1. To facilitate future constructions an Xba I site was engineered immediately upstream of the first ATG initiation codon of this clone by oligonucleotide mediated mutagenesis by the method of Kunkel using the oligonucleotide:

5′-TACCAACCATCGGCGTCTAGAGGCAATGGCGGC-3′

producing plasmid pMON9950. pMON9950 was digested with Xba I and religated to eliminate the 126 bp Xba I fragment at the 5′ end of the cDNA forming pMON9951. To produce a coding sequence which encodes for a glyphosate tolerant form of maize EPSP synthase, pMON9951 was mutated by the method of Kunkel using the oligonucleotide:

5′-CTTCTTGGGGAATGCTGCTACTGCAATGCGGC-3′

resulting in pMON9960. This mutagensis will change a GGA codon to a GCT codon, changing the second glycine residue in the conserved sequence -L-G-N-A-G-T-A- to an alanine in the resulting protein. The glycine residue is amino acid 163 of the predicted maize preEPSP synthase. This would correspond to amino acid 95-105 of the mature protein depending on the precise transit peptidase cleavage site which has not been determined. pMON9960 was then mutagenized by the same method with the oligonucleotide.

5′-TCGGATTGAAGCAGCTTGACGCAGATGTTGAT-3′

resulting in the formation of pMON8617, which would include the aspartate for glycine substitution at position 206 of the maize EPSP synthase precursor. This would correspond to a position between amino acid residues 139 and 149 of the mature protein.

To demonstrate that this alteration produced a glyphosate tolerant form of maize EPSP synthase, protein was produced from pMON9951 and pMON8617 by *in vitro* transcription with T7 RNA polymerase, followed by *in vitro* translation as follows: Plasmid DNA (pMON9951 and pMON8617) containing the full-length EPSP synthase cDNA was linearized with EcoRI. The linearized plasmid DNA was transcribed *in vitro* with T7 polymerase essentially as described by Krieg et al. (1984). The standard reaction buffer contained 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 80 U RNAsin ribuonuclease inhibitor, 0.5 mM each of ATP, GTP, CTP and UTP, in a final reaction volume of 100 µl. The final RNA pellet was resuspended in 20 µl of sterile water and stored at -80° C. A standard translation reaction contained 100 µl of nuclease-treated rabbit reticulocyte lysate, 5.7 µl of a 19-amino acid mixture (minus methionine) at 1 mM each, 5.7 µl of RNA (total RNA transcripts derived from 0.63 µg of plasmid DNA), 16 µl RNAsin (20U/µl) ribonuclease inhibitor, and 58.3 µl of [^{35}S] methionine (14-15 mCi/ml). The *in vitro* translation products were stored frozen at -80° C.

The products of the *in vitro* translation were then assayed for EPSP synthase activity as described herein. The product of pMON8617 showed detectable EPSP synthase activity in the absence of glyphosate. When the assay was repeated in the presence of 1.0 mM glyphosate no activity was detected. In contrast the variant preenzyme product of pMON8617 showed a high level of tolerance to glyphosate, showing only slight inhibition at 1 mM glyphosate, 25% inhibition at 10 mM glyphosate and greater inhibition, but still showing detectable activity at 100 mM glyphosate.

For expression in maize cells the coding sequence of the glyphosate tolerant variant form of maize pre-EPSP synthase is excised from pMON9960 and inserted between a promoter known to function in maize cells, such as the CaMV35S promoter, and the poly A addition site of the nopaline synthase gene or another suitable gene. In addition, an intron such as the first intron of the maize ADH1 gene may be included in the 5′-untranslated region of the expression unit which may enhance expression of the chimeric gene (Callis et al., 1987).

One such vector was constructed as follows: the plasmid pUC119 (described hereinbefore) was digested with Eco RI and Hind III. The synthetic DNA fragment:

```
5'-AATTGCGGCCGCGRRAACTGCAGCCCGGGCGGCCGC-3'
    3'-CGCCGGCGCAATTGACGTCGGGCCCGCCGGCGTCGA-5'
```

was inserted into the digested plasmid to create pMON914. Through a series of standard cloning steps the enhanced version of the 35S promoter (Kay et al. 1987) a multilinker sequence, and 3′-end of the nopaline synthase gene were inserted into plasmid pMON914 to form pMON9948. A 557 bp BclI/BamHI fragment containing the first intron of the maize ADH1 gene (Callis et al. 1987) was inserted into the BglII site of pMON9948 to form pMON9955. For expression in maize cells and plants the coding sequence of the variant EPSP synthase was removed from pMON8617 on an Xba I/EcoRI fragment and inserted into pMON9955 which had been digested with XbaI and EcoRI resulting in pMON8631 (Figure 7). This plasmid contains an expression unit consisting of the enhanced version of the CaMV 35S promoter, the first intron of the maize ADH1 gene (in the 5′ untranslated sequence), the maize preEPSP synthase cDNA with the glycine to alanine and glycine to aspartate changes and the nopaline synthase 3′-end.

Transgenic maize cells can be prepared by bombarding maize cells, such as the suspension line BMS1 (ATCC 54022), with particles coated with pMON8631 by the method of Klein et al. (1988) or the method of Christou et al. (1988). The cells are then selected for 1-3 weeks in medium containing 5 mM glyphosate followed by selection on solid medium containing 5 mM glyphosate. Calli which have incorporated and are expressing the chimeric variant EPSP synthase gene can be identified by their rapid growth on the solid medium.

Alternatively the EPSP synthase expression unit is inserted into a vector which includes the neomycin phosphotransferase gene under control of the CaMV35S promoter, or a similar vector with a different marker gene that allow for a selection of transformed maize cells. This vector, or a similar vector using any other glyphosate resistant coding sequences constructed as described in the claims and examples of this application, is then introduced into maize cells as described in the following example.

Preparation of Maize Protoplasts

Protoplasts are prepared from a Black Mexican Sweet (BMS) maize suspension line, BMSI (ATCC 54022) as described by Fromm et al. (1985 and 1986). BMSI suspension cells are grown in BMS medium which contains MS salts, 20 g/l sucrose, 2 mg/l 2,4-dichlorophenoxy acetic acid, 200 mg/l inositol, 130 mg/l asparagine, 1.3 mg/l niacin, 0.25 mg/l thiamine, 0.25 mg/l pyridoxine, 0.25 mg/l calcium pantothenate, pH 5.8. 40 ml cultures in 125 erlenmeyer flasks are shaken at 150 rpm at 26° C. The culture is diluted with an equal volume of fresh medium every 3 days. Protoplasts are isolated from actively growing cells 1 to 2 days after adding fresh medium. For protoplast isolation, cells are pelleted at 200 X g in a swinging bucket table top centrifuge. The supernatant is saved as conditioned medium for culturing the protoplasts. Six ml of packed cells are resuspended in 40ml of 0.2 M mannitol/50 mM CaCl2/10 mM sodium acetate which contains 1% cellulase, 0.5% hemicellulase and 0.02% pectinase. After incubation for 2 hours at 26° C, protoplasts are separated by filtration through a 60 um nylon mesh screen, centrifuged at 200 X g and washed once in the same solution without enzymes.

Transformation of Maize Protoplasts Using an Electroporation Technique

Protoplasts are prepared for electroporation by washing in a solution containing 2 mM potassium phosphate pH 7.1, 4 mM calcium chloride, 140 mM sodium chloride and 0.2 M mannitol. After washing, the protoplasts are resuspended in the same solution at a concentration of 4 X 10E6 protoplasts per ml. One-half ml of the protoplast containing solution is mixed with 0.5 ml of the same solution containing 50 micrograms of supercoiled plasmid vector DNA and placed in a 1 ml electroporation cuvette. Electroporation is carried out as described by Fromm et al. (1986). As described, an electrical pulse is delivered from a 122 or 245 microFarad capacitor charged to 200 V. After 10 minutes at 4° C and 10 min at room temperature protoplasts are diluted with 8 ml of medium containing MS salts 0.3 M mannitol, 2% sucrose, 2 mg/l 2,4-D, 20% conditioned BMS medium (see above) and 0.1% low melting agarose. After 2 weeks in the

dark at 26° C, medium without mannitol and containing kanamycin is added to give a final concentration of 100 mg/l kanamycin. After an additional 2 weeks, microcalli are removed from the liquid and placed on a membrane filter disk above agarose-solidified medium containing 100 mg/l kanamycin. Kanamycin resistant calli composed of transformed maize cells appear after 1-2 weeks.

Glyphosate tolerant maize cells

As described by Fromm et al. (1986), transformed maize cells can be selected by growth in kanamycin containing medium following electroporation with DNA vectors containing chimeric kanamycin resistance genes composed of the CaMV35S promoter, the NPTII coding region and the NOS 3' end. These cells would also be producing the glyphosate tolerant form of EPSP synthase and would tolerate elevated levels of glyphosate.

The electroporated cells could also be selected as described above by transferring them directly into glyphosate containing liquid medium followed by selection on solid medium containing glyphosate.

Alternative methods for the introduction of the plasmids into maize, or other monocot cells would include, but are not limited to, the injection method of Newhaus et al. (1987), the injection method of de la Pena et al. (1987) or the microprojectile methods of Klein et al. (1987) and McCabe et al. (1988).

The embodiments described above are provided to better elucidate the practice of the present invention. It should be understood that these embodiments are provided for illustrative purposes only, and are not intended to limit the scope of the invention.

BIBLIOGRAPHY

Adams, S. P. and Galluppi, G. R. (1986) Medicinal Research Reviews 6:135-170.

Adams, S. P., et al., (1983) J. Amer. Chem. Soc. 105:661.

Ausubel, F., et al., (1980) Plant Mol. Bio. Newsletter 1:26-32.

Bachman, B. J., et al., (1980) Microb. Rev. 44:1-56.

Brau, et al., Mol. Gen. Genet. 193:179-187 (1984).

Broglie, R., et al., (1983) Bio/Technology 1:55-61.

Broglie, R., et al., (1984) Science 224:838-843.

Callis, J., Fromm, M. and Walbot, V. (1987) Genes and Develop. 1:1183-1200.

Charles, G. Keyte, J. W., Brammer, W. J., Smith, M. and Hawkins, A. R. (1986) Nucleic Acids Res. 14:2201-2213.

Covey, S., Lomonosoff, G. and Hill, R., (1981) Nucleic Acids Res. 9:6735.

de la Pena, A., Lorz, H. and Schell, J. (1987) Nature 325:274-276.

Depicker A., et al., (1982) J. Mol. Appl. Gen. 1:561.

Ditta, G., et al., (1980) Pro. Natl. Acad. Sci. USA 77:7347.

Duncan, K., Lewendon, A. and Coggins, J. R. (1984) FEBS Lett. 170:59-63.

Fraley, R. T., Rogers, S. G., Horsch, R. B., Eichholtz, D. A., Flick, J. S., Fink, C. L., Hoffmann, N. L. and Sanders, P. R. (1985) Bio/Technology 3:629-635.

Fromm, M., Taylor, L. P. and Walbot, V. (1985) Proc. Nat. Acad. Sci. USA 82:5824-5828.

Fromm, M., Taylor, L. P. and Walbot, V. (1986) Nature 319:791-793.

Gardner, R., Howarth, A., Hahn, P., Brown-Luedi, M., Shepherd, R. and Messing, J., (1981) Nucleic Acids Res. 9:2871.

Gritz and Davies, Gene 25:179-188 (1984).

Guilley, H., Dudley, R., Jonard, G., Balax, E. and Richards, K. (1982) Cell 30:763.

Goldberg, R. B., et al., (1981) Devel. Bio. 83:201-217.

Gubler, U., and Hoffman, B. H. (1983) Gene 25:263-269.

Horsch, R. and Klee, H., (1986) Proc. Natl. Acad. Sci. USA vol. 83, 4428-4432.

Horsch, R. B., and Jones, G. E. (1980) In Vitro 16:103-108.

Horii, T., et al. (1980) P.N.A.S. USA 77:313.

Hunkapiller, M. W., et al. (1983b) Methods Enzymol. 91:486-493.

Huynh, T. V. Young, R. A. and Davis, R. W. (1985) DNA Cloning Techniques: A Practical Approach , D. Glover ed., IRL Press, Oxford.

Kay, R., Chan, A., Daly, M., and McPherson, J. (1987) Science 236:1299-1302.

Klein, T. M., Wolf, E. D., Wu, R. and Sanford, J. C. (1987) Nature 327:70-73.
Krieg, P. A. and Melton, D. A. (1984) Nucleic Acids Res. 12:7057-7070.
Kunkel (1985) P.N.A.S. USA 82:488-492.
Lemke, G. and Axel, R. (1985) Cell 40:501-508.
Maniatis, T., et al. (1982) Molecular Cloning: A Laboratory Manual , Cold Spring Harbor Labs., NY.
Marmur, J. (1961) J. Mol. Biol. 3:208-210.
Maskell, D. J. et al. (1988) J. Bacteriology 170,6:2467-2471.
Maxam, A. M. and Gilbert, W., (1977) P.N.A.S. USA 74:560-564.
McCabe, D. E., et al. (1988) Bio/Technology 6:923.
Messing, J. et al. (1981) Nucleic Res. 9:309-321.
Mousdale, D. M. and Doggins, J. R. (1984) Planta 160:78-83.
Neuhaus, G., et al. (1987) Theor. Appl. Genet 75:30.
Okayama, H. and Berg, P. (1982) Mol. Cell. Biol. 2:161.
Padgette, S. R., et al. (1987) Arch. Biochem. Biophys. 258:564-573.
Rao, R. N. and Rogers, S. G. (1979) Gene pp. 7:79-82.
Rochester, D. E., Winter, J. A. and Shah, D. M. (1986) EMBO J. 5:452-458.
Rogers, S., Horsch, R. and Fraley, R., (1986) Methods in Enzymology Vol. 118 (H. Weissbach and A. Weissbach, eds.) p.627, Academic Press, New York.
Rogers, S. G., et al. (1983) Appl. Envir. Microbio. 46:37-43.
Rogers, S., Klee, H., Horsch, R. and Fraley, R. (1987) Methods in Enzymology Vol. 153 (R. Wu and L. Grossman, eds.) p.253, Academic Press, New York.
Sancar, A., et al. (1980) P.N.A.S. USA 77:2611.
Sanger, et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463.
Scherer, et al. (1981) Developmental Bio. 86:438-447.
Schimke, R. T. ed. (1982) Gene Amplification , Cold Spring Harbor Labs.
Schmidhauser, T. and Helinski, D. (1985) J. Bacteriology 164:446.
Schuler, M. A., Schmitt, E. S. and Beachy, R. N. (1982) Nucl Acids Res. 10:8225-8244.
Soberson, et al. (1980), Gene 9:287-305.
Southern, E. M. (1975) J. Mol. Biol. 98:503-517.
Stalker, D. M., Hiatt, W. R. and Comai, L. (1985) J. Biol Chem. 260:4724-4728.
Steinrucken, H. and Amrhein, N. (1980) Biochem. & Biophys. Res. Comm. 94:1207-1212.
Vieira, J. et al., (1982) Gene 19:259-268.
Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33:103-119.
Zoller, M. M. et al. (1983) Methods Enzymol. 100:468.

Claims

1. A method for producing glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthase enzymes which comprises substituting an alanine residue for the second glycine residue in a first amino acid sequence:
-L-G-N-A-G -T-A-
located between positions 80 and 120 in a mature wild type EPSP synthase sequence, and substituting an amino acid residue selected from the group consisting of aspartic acid and asparagine for the terminal glycine residue in a second amino acid sequence:
E-R-P-I-x_1-x_2-L-V-x_3-x_4-L-x_5-x_6-x_7-G -A-
where x_1,x_2,x_3,x_4,x_6 and x_7 are any amino acid residue, and x_5 is either arginine or lysine, said second amino acid sequence located between positions 120 and 160 in the mature wild type EPSP synthase sequence.

2. A method of Claim 1 in which the glyphosate-tolerant EPSP synthase is produced from a wild-type plant EPSP synthase.

3. A method of Claim 1 in which the glyphosate-tolerant EPSP synthase is produced from a wild-type bacterial EPSP synthase.

4. A method of Claim 1 in which the glycine residue of the second amino acid sequence is replaced with an aspartic acid residue.

5. A glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthase enzyme which contains a first amino acid sequence:
-L-G-N-A-A-T-A-

between positions 80 and 120 in the mature EPSP synthase sequence, and a second amino acid sequence:

$$E\text{-}R\text{-}P\text{-}I\text{-}X_1\text{-}X_2\text{-}L\text{-}V\text{-}X_3\text{-}X_4\text{-}L\text{-}X_5\text{-}X_6\text{-}X_7\text{-}\overset{D}{N}\text{-}A\text{-}$$

where x_1, x_2, x_3, x_4, x_6, and x_7 are any amino acid residues and x_5 is either arginine or lysine, said second amino acid sequence located between positions 120 and 160 in the mature EPSP synthase sequence.

6. A glyphosate-tolerant EPSP synthase enzyme of Claim 5 as shown in Figure 1.

7. A glyphosate-tolerant EPSP synthase of Claim 5 produced by the method of Claim 2 wherein the wild-type EPSP synthase coding sequence is selected from the group of EPSP synthases consisting of petunia, tomato, maize, *Arabidopsis thaliana*, soybean, *Brassica napus*, *E. coli* K-12, *Bordetella pertussis* and *Salmonella typhimurium* as shown in Figure 1.

8. A plant gene encoding a glyphosate-tolerant EPSP synthase enzyme of Claim 5.

9. A plant transformation vector comprising a gene which encodes a glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate (EPSP) synthase having a first amino acid sequence:
-L-G-N-A-A-T-A-
located between positions 80 and 120 of the mature EPSP synthase sequence and a second amino acid sequence:

$$E\text{-}R\text{-}P\text{-}I\text{-}X_1\text{-}X_2\text{-}L\text{-}V\text{-}X_3\text{-}X_4\text{-}L\text{-}X_5\text{-}X_6\text{-}X_7\text{-}\overset{D}{N}\text{-}A\text{-}$$

where x_1, x_2, x_3, x_4, x_6, and x_7 are any amino acid residues and x_5 is either arginine or lysine, said second amino acid sequence located between positions 120 and 160 in the mature EPSP synthase sequence.

10. A vector of Claim 9 containing a glyphosate-tolerant plant EPSP synthase.

11. A vector of Claim 9 containing a glyphosate-tolerant bacterial EPSP synthase.

12. A vector of Claim 9 containing a glyphosate-tolerant fungal EPSP synthase.

13. A transformed plant cell containing a gene of Claim 8.

14. A transformed plant cell of Claim 13 selected from cells of tomato, tobacco, oil seed rape, flax, soybean, sunflower, sugar beet, alfalfa, cotton, rice and maize.

15. A plant comprising transformed plant cells of Claim 13.

16. A plant of Claim 15 in which the plant is selected from tomato, tobacco, oil seed rape, flax, sunflower, sugar beet and alfalfa.

17. A seed produced by a plant of Claim 15.

18. A seed produced by a plant of Claim 16.

19. A method for producing glyphosate-tolerant plants which comprises propagating a plant containing a plant gene of Claim 5.

20. The method of Claim 19 in which the plant is selected from maize, tomato, tobacco, oil seed rape, flax, sunflower, sugar beet, alfalfa, cotton, and rice.

21. A method of Claim 19 in which a first plant is propagated by crossing between said first plant and a second plant, such that at least some progeny of said cross display glyphosate tolerance.

22. A method of Claim 21 in which the plant is selected from maize, tomato, tobacco, oil seed rape, flax, sunflower, sugar beet, alfalfa, cotton and rice.

23. A DNA sequence encoding a glyphosate-tolerant EPSP synthase of Claim 5.

24. A DNA sequence of Claim 23 which is less than twenty kilobases in length.

25. A DNA sequence of Claim 24 encoding a glyphosate-tolerant EPSP synthase of Claim 6.

26. A DNA sequence of Claim 23 encoding a glyphosate-tolerant EPSP synthase of Claim 7.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

DOCUMENTS CONSIDERED TO BE RELEVANT

EP 90870111.3

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	EP - A2 - 0 293 358 (MONSANTO COMP.) * Claims 1-3,5,7-14,24-47 *	1-3,5, 7-26	C 12 N 15/54 C 12 N 9/10 C 12 N 15/82 C 12 N 5/04 A 01 H 1/00 C 07 H 21/04
D,A	EP - A2 - 0 218 571 (MONSANTO COMP.) * Claims *	1,5,8, 9,13-20,23	
A	EP - A2 - 0 115 673 (CALGENE, INC.) * Abstract *	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 N
A 01 H
C 07 H

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	18-10-1990	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)